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THE EFFECT OF TOXAEMIA ON METABOLISM

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The recent progress made in biochemistry, which has depended as well upon the elaboration of new technique, as upon the intensive use of classical methods of investigation, has of recent years made it possible to approach some of the problems of pathology from a new angle. Hitherto, except in the case of certain diseases clearly recognized as essentially "metabolic," such as diabetes, pathological changes have, for the most part, been recognized and described in terms of morbid histology. In conditions in which histological methods can be employed, they have often proved of the utmost value in diagnosis; indeed modern clinical medicine rests upon the foundations of morbid anatomy and morbid histology. Yet anatomical and histological investigation reveals changes in form without necessarily indicating how far such changes may be accompanied by an alteration in function. It may be obvious that the major part of an organ has been destroyed, and replaced by fibrous tissue; and such a finding may be correlated with a clinical picture which is dominated by gross insufficiency in the function of that organ. But such a condition, when far enough advanced to be clearly recognized is often irremediable. On the other hand, material is often available to show that in an acute illness, there are definite changes in cells which can regularly be demonstrated histologically, and which, it is fair to assume, go on, in non-fatal cases, not to destruction and replacement by fibrous tissue, but to repair. The question arises as to what disturbance in function accompanies these lesions. Since the patient is clinically "ill" and yet complete recovery occurs, it is plain that bodily function has been temporarily disturbed. The exactness with which such disturbance can be described, depends, equally obviously, on our knowledge of normal function. In the case of the circulatory system, disturbances can probably be most accurately

described in terms of haemodynamics and electrophysiology. On the other hand, knowledge of the metabolism of organs, such as the liver and the kidney, and perhaps the central nervous system, is increasing so rapidly, that in these cases pathological changes may soon be most readily and most usefully explicable in biochemical terms. Indeed, it seems that the beginnings of such a "chemical pathology" already exist; and, trivial though they are in comparison to the importance which they will doubtless assume in the future, it is the object of this review to gather them together as far as may be possible. Such studies must obviously depend for their inception and inspiration on clinical observation and on the pathological findings in human cases. In many instances they must depend on animal experiment if they are to be carried very far.

It is not intended to review the vast literature which has gathered round the study of diabetes and nephritis. These were recognized from the first as diseases which must be described in chemical terms, and have been investigated by chemical methods. But there is little doubt that the existence of diabetes forced the development of the first satisfactory "micro" method, that for blood sugar, and so has influenced the whole trend of modern biochemistry which depends so largely on accurate "micro" determinations of various substances.

Hibbard and Morrisey (1899) recognized the occurrence of glycosuria in patients suffering from diphtheria, but one of the earliest experimental studies of the effect of toxaemia upon carbohydrate metabolism was made by Rosenthal (1914). He used rabbits, and injected large doses (lethal in 12–48 hours) of diphtheria toxin intravenously. He observed a fall in blood sugar. He noted a fall in temperature, in blood pressure, and a terminal "myasthenia," though he made no mention of convulsions. He discussed at length the previous literature bearing on the morbid histology and adrenalin content of the suprarenal glands in diphtheria, and drew a parallel between the symptoms of suprarenalectomy and those of diphtheritic toxaemia.

He shows that, even if rabbits are given a very high carbohydrate diet before receiving toxin, their livers contain little or no glycogen when they are killed. He argues that, since glycogen disappears without giving rise to a hyperglycaemia, the loss of glycogen cannot be due merely to accelerated glycogenolysis, but must be caused by a failure of the liver to synthesise glycogen. He observes that, after the administration of glucose the hyperglycaemia is more prolonged, and the blood sugar rises more than in normal animals. In late toxaemia,

adrenalin fails to raise the blood sugar. Though the conception of gluconeogenesis was less fully developed than it is at present, yet it is clear that Rosenthal had grasped the idea that in severe toxaemia, the power of the liver to manufacture carbohydrate was impaired. He stressed the probable rôle of suprarenal lesions in the general picture.

Hector (1926) conducted a clinical investigation on diphtheria patients. He observed a "diabetic" type of glucose tolerance curve, with (in severe cases) a low fasting blood sugar value. The urine was acid, the "ammonia coefficient" low, and laevulose tolerance tests indicated some liver damage. The respiratory quotient failed to rise after glucose ingestion. He discusses the probability of endocrine lesions in diphtheritic toxaemia, and notes that two of his cases which proved to be suffering from quinsey, and not from diphtheria, showed no disturbance of glucose tolerance, though they did show an abnormal laevulose tolerance.

Elkeles and Heimann (1927a, b) investigated the blood sugar in cases of human diphtheria. In some of their severe cases they found a low fasting blood sugar, but in others it was normal or high. Some cases gave glucose tolerance tests of a diabetic type, and similar curves were found in cases of diphtheritic paralysis. The ingestion of 40 grams of fructose sometimes gave rise to hyperglycaemia and glycosuria, and they demonstrated insulin resistance in some of their cases, though if the insulin were previously incubated with diphtheria toxin, its power to lower the blood sugar of rabbits was unimpaired. They discussed the possibility that diphtheria intoxication had a "sympatheticotonic" effect, and they showed that ergotamine tartrate largely abolished the rise of blood sugar which followed the ingestion of glucose by their diphtheritic patients.

Mikami (1925) injected very large doses of diphtheria toxin into rabbits. Death followed in most cases within 24 hours. He found an initial hyperglycaemia, followed by a fall in blood sugar, and death in hypoglycaemia. The adrenalin content of the suprarenal medulla fell slightly at first, rose, and fell again. The liver glycogen fell markedly. The decrease both in adrenalin and in liver glycogen was prevented by double splanchnotomy.

Stimulated by often repeated observation of the adverse effects of intercurrent infections upon the course of diabetes, and upon its treatment with insulin, Lawrence and Buckley (1927) investigated the effect of experimental diphtheritic toxaemia upon the response of rabbits to an injection of insulin. The dose of toxin was of a size to

cause death in from seven to eight days in the majority of the animals. It was regularly observed that, during toxaemia, the response to insulin was diminished, and in some cases was altogether abolished. In some animals which survived for seven or eight days, a "pre-lethal" rise of temperature was observed and this was accompanied by a marked hyperglycaemia, though in animals which died in one or two days, a hypoglycaemia was usual. The hyperglycaemia was abolished by injections of ergotamine tartrate, and was therefore ascribed to over-activity of the sympathetico-adrenal system. The authors attribute the resistance to the hypoglycaemic action of insulin to increased glyconeogenesis caused by the stimulation by the toxin of the thyroid and pituitary glands, and they discuss the clinical evidence pointing to a like cause of the disturbances of carbohydrate metabolism met with both in normal and diabetic subjects in infective states. Cramer (1926) had already pointed out that the "thyro-adrenal apparatus" played a large part in the control of body temperature, and had brought together much evidence that lesions of the thyroid and adrenals are regularly found in infective conditions.

Zweckwer and Goodell (1925a) observed, confirming the observation of Menton and Manning (1924), that the injection of heat-killed cultures of certain organisms produced a marked hyperglycaemia in rabbits. The hyperglycaemia produced by similar killed cultures of some other organisms was, by comparison, very slight, while a third group produced no rise in blood sugar at all. The organisms in the first class were B. proteus, B. coli, and B. paratyphosus B; in the second, B. paratyphosus A, and B. enteritidis; and in the third, B. faecalis alkaligenes, Strep. haemotyticus, Strep. viridans, Staph. aureus, B. pyocyaneus, and B. typhosus. Severe symptoms were frequently observed, but were not apparently related to the hyperglycaemia.

Sweeney and Lackey (1928) used rabbits into which they injected a dose of diphtheria toxin which was usually lethal in from five to seven days. Dextrose tolerance tests were carried out by giving by stomach tube 5 grams of glucose dissolved in 25 cc. of water. As the toxaemia progressed, the tolerance of the animals became more and more impaired. After three to four days' toxaemia, the blood sugar two hours after the administration of the glucose sometimes reached 400 to 500 mgm. per 100 cc. of blood, and the curve showed no signs of flattening or dropping at any point. The authors suggest that there is a quantitative relationship between the severity of the toxaemia and the impairment of the glucose tolerance, due to a disturbance either of the

action, or of the production, of insulin. Sweeney (1928) found that the injection of insulin at the beginning of the glucose tolerance test caused a drop in blood sugar, or at least a flattening of the curve. In spite of the insulin, the tolerance was much impaired in toxaemic animals, but it was argued that since the action of exogenous insulin can be demonstrated, the effect must be due to a failure in endogenous insulin production.

Sweeney, Barshop and LoBello (1934), however, on extending these investigations, found that, as toxaemia progressed day by day, more and more insulin had to be given with the standard dose of glucose to keep the glucose tolerance curve within normal limits. The amount of insulin might rise from 2 units on the first day to 12 or 14 on the 5th. Sweeney, Barshop, LoBello and Rosenthal (1934) found that in normal animals the form of the glucose tolerance curve could be kept constant even when the dose of glucose was considerably increased, provided that it was accompanied by a proportionately increased dose of insulin. After diphtheria toxin, however, this was no longer the case; the blood sugar rose much higher as the dose of glucose was increased, despite the extra insulin. These further findings were interpreted as showing that the effect of exogenous, as well as endogenous insulin was diminished in toxaemia.

Schwenkter and Noel (1930), also using rabbits, and employing a similar dose of diphtheria toxin to that used by Sweeney found that the terminal blood sugar of those rabbits that survived more than 70 hours after the administration of the toxin was higher than before toxin was given, while terminal hypoglycaemia occurred in animals surviving less than 70 hours. The figures for terminal blood sugar, when plotted against survival time, give a remarkably straight line. The animals dying early had terminal convulsions, those dying late were usually in coma for some time before death. A few determinations of liver glycogen gave very low values in all cases. Human patients (children) showed diminished dextrose tolerance and glycosuria after injections of glucose; insulin lowered the blood sugar curve, but the doses used did not bring it back within normal limits.

Schwenkter and Noel (1930b) treated severely toxic cases of diphtheria with insulin and glucose. As large doses of antitoxic serum were also given, the results are of little value.

Evans and Zweckwer (1927) investigated further the hyperglycaemia which occurs after the injection of killed cultures of B. coli and B. Proteus. They found it to be a regular phenomenon; but the rise in

blood sugar could be neutralized by insulin, and the phenomenon was abolished by ergotamine tartrate. They found that if one suprarenal was removed and the other denervated some time before the injection, the hyperglycaemic response was replaced by a hypoglycaemia, which was sometimes fatal. On these grounds they concluded that the hyperglycaemia was due to a stimulation of the suprarenals, which caused a mobilization of sugar from the liver. Thyroid feeding tended to increase the hyperglycaemia, thyroidectomy to diminish it.

In these experiments, as in those of Zweckwer and Goodell, the hyperglycaemic agent was presumably some unknown but heat stable constituent present in the bodies of some (but not of all) bacteria. Zweckwer and Goodell (1925b) found that bacterial anaphylaxis induced in rabbits by injection of killed organisms always resulted in a marked hyperglycaemia, which in one case reached a value of more than 1,000 mgm. per 100 cc. of blood. This phenomenon occurred with all the kinds of bacteria tested, and was therefore independent of the hyperglycaemia which occurs when single injections of some kinds, but not of others are given.

Benn, Hughes and Alstead (1932) showed that the response to the intravenous injection of 20 grams of glucose into human diphtheria patients resulted in a glucose tolerance curve progressively more "diabetic" in type as the cases were more severe. The curve returned to normal as the condition improved, but reverted to the diabetic type if cardiac or nervous sequelae developed. Insulin brought the curves back towards the normal type, and treatment of severe cases with glucose and insulin, as well as with anti-toxic serum was tried, with some apparent success when the results were compared with a previous series of cases which had received serum only.

Williams and Dick (1932) looked for glycosuria in a number of human cases of acute infectious disease. In 41 per cent of the cases it was found; it was usually reduced by insulin administration. It was accompanied by diminished dextrose tolerance, and the tolerance was improved by giving insulin. Most patients examined were suffering from scarlet fever, but the series also included examples of diphtheria, pneumonia, influenza, tonsillitis, measles, erysipelas, encephalitis, mumps, epidemic meningitis, and poliomyelitis. They found diminished dextrose tolerance in rabbits after the intravenous injection of 48 hour cultures of the following organisms: Streptococcus of scarlet fever, Pneumococcus type I, Pneumococcus type III, B. typhosus, B. paratyphosus B, B. influenzae, Streptococcus of erysipelas, scarlet fever anaerobic organisms.

Corkill (1932) observed that injections of diphtheria toxin abolished the power, possessed by normal young rabbits, to lay down glycogen in the liver in response to an injection of insulin or of adrenalin. This occurred even after toxaemia of short duration, when no histological lesions were visible in the liver and when the injection of glucose still gave rise to a deposition of liver glycogen. Toxaemia of 24 hours' duration also made the animals resistant to insulin. After a dose which would normally cause convulsions the blood sugar fell to about 60 mgm. per 100 cc., but then rose again spontaneously, and would do so several times in succession in response to repeated doses of insulin. Adrenalin at this stage of toxaemia produced somewhat less hyperglycaemia than in a normal animal. T. R. Elliott (1912) found that, provided that the splanchnic nerves were intact, diphtheritic toxaemia lowered the adrenalin content of the suprarenals. Corkill found that, histologically, the glands showed loss of chromaffine tissue, which progressed with the severity of the toxaemia; while the organs did not show obvious signs of damage, their adrenalin content was diminished. Since the insulin resistance was abolished by ergotoxin, it was interpreted as being due to an increased secretion of adrenalin.

Corkill and Ochoa (1934) found that while lactate, injected intravenously into young rabbits which had been starved for 24 hours, gave rise to a definite deposition of liver glycogen, no such deposition occurred if the animals had been injected 24 hours previously with diphtheria toxin. Since they did not detect any impairment in the power of muscle extract made from such rabbits to form lactic acid from glycogen or from hexose diphosphate, and since glycogen was still deposited in their livers after an injection of *glucose*, they concluded that the power of the liver to form glycogen from lactate was impaired; they thought that this might be due to a direct effect on the liver cells, but did not exclude an effect on the suprarenal cortex.

Cross and Holmes (1937) were able to confirm that, in vivo, little glycogen could be deposited after an injection of lactate into a 24 hour toxaemic rabbit; they further noticed that, although glycogen was still deposited after an injection of glucose, the amount was distinctly less than in a normal animal after a similar injection.

Soskin, Allweiss and Mirsky (1935) investigated the dextrose tolerance curves of normal and depancreatized dogs an hour or so after a massive intravenous injection of diphtheria toxin. Soskin and his collaborators had previously developed the thesis that the form of the normal dextrose tolerance curve depended on the "homoeostatic" power of the liver rather than upon a secretion of insulin evoked from

the pancreas to deal with hyperglycaemia, and they had shown that normal dextrose tolerance curves could be obtained in depancreatized dogs[1] if the test injection of glucose were superimposed upon a steady intravenous infusion of glucose and insulin so adjusted as to keep the blood sugar level constant. (Soskin, Allweiss and Cohen, 1934; Soskin and Allewiss, 1934; see also Soskin, Essex, Herrick and Mann, 1939). They therefore argue that an important factor in the mechanism for blood sugar regulation is the power of the liver cells to regulate their intake or output of sugar in accordance with the blood sugar level at any moment ("homoeostatic" power). After the injection of diphtheria toxin, the normal, and still more the depancreatized dogs showed a grossly diabetic type of sugar tolerance curve, even though in both cases control curves, obtained immediately before the injection of toxin, were of the usual form. They inferred from this that the liver cells were being affected by the toxin, which, they believed, altered the sensitivity of the liver to the control of the endocrine secretions.

In a further paper Soskin and Mirsky (1935) investigated the effects of rather smaller doses of diphtheria toxin (lethal in about 10 hours) administered to dogs. When no glucose was given there was a slow rise, with a terminal sharp decline in blood sugar; the animals died in hypoglycaemia. If glucose tolerance tests were performed, the results depended to some extent upon the size of the dose of glucose administered. The first change was the appearance of a "diabetic" type of curve, caused, the authors believe, by a derangement of the "homoeostatic" mechanism in the direction of increased glycogenolysis. This might be followed by the reappearance of a normal curve. In the later stages, the curve was of an extreme "diabetic" type. Similar curves are seen after hepatectomy, and so probably correspond to a complete failure of liver function, which, had no glucose been given, would have resulted in a severe hypoglycaemia. It is of interest that the authors do not record that the administration of glucose prolonged the survival time of the animals.

It will be clear from the work which has been discussed that there

[1] In connection with the theory of the "homoeostatic" mechanism of the liver, it is worth remarking that, while Zunz and La Barre (1927) used dogs, many of the experiments which have given support to the theory that the blood sugar is controlled by nervous influences acting on the pancreas via the vagus nerves have been carried out on the rabbit. Hoet found that sections of the vagi in the dog, without or with removal of the kidneys, failed to modify the glucose tolerance curve as it does in rabbits. It may therefore be that the "homoeostatic" mechanism is more active in the dog than in other animals.

is abundant evidence that in toxaemia, whether occurring in human cases or produced experimentally in animals, there is a disturbance, more or less severe, of the normal metabolism of carbohydrate. The knowledge that diabetes, the classical example of disturbed carbohydrate metabolism, was due to deficiency of an internal secretion of the pancreas, and the discovery of the effects of adrenalin upon the blood sugar level, has naturally disposed workers to seek for an explanation of their results in an effect of the toxin upon one or more of the endocrine glands, the more so since there is a very extensive literature dealing with the histological changes observed in different endocrine organs in toxaemic conditions. (For references see Elliott, 1912; Rosenthal, 1914; Cramer, 1926; Elkeles and Heimann, 1927.) While a discussion of this aspect of the matter is beyond the scope of this review, there is not the smallest doubt but that lesions which can easily be recognized histologically appear in the endocrine glands at an early stage in many toxaemic conditions. It is, however, only very recently that it has been possible to appreciate how complicated is the endocrine control of metabolism. The earlier workers in the field were not able to visualise very much more than a disturbance, in the direction either of increased or decreased secretion, of the adrenal medulla, the islets of Langerhans, or the thyroid; for the functions of the adrenal cortex, the anterior lobe of the pituitary, and the parathyroids were almost unknown. We therefore propose to discuss (as far as may be in the light of recent developments) the evidence that the metabolic changes observed in toxaemia are caused by damage to the endocrine glands, without prejudicing the question whether such damage is, in fact, the sole or most fundamental cause, or whether damage to such organs as the liver does not also play a very important part.

Recent work upon the function of the anterior pituitary and the suprarenal cortex has profoundly modified our conception of diabetes, and it is clear that the effects of toxaemia must be interpreted if possible in the light of this knowledge. All observers agree that, in toxaemia, the glucose tolerance curve is modified in the sense that after a standard dose of glucose, administered either by mouth or intravenously, the blood sugar shows a more pronounced and more prolonged rise than usual—that, in fact, the tolerance curve becomes more "diabetic" in type. Clearly enough, this could result from one or more than one of a number of different causes: a failure in the utilization of glucose, a failure in its storage, or a failure in some mechanism by which gluconeogenesis is normally checked when glucose is introduced into the

blood stream from without (this might correspond to the "homoeostatic" mechanism of Soskin). Any or all of these mechanisms may be controlled by the endocrine glands, and may be affected by damage which falls primarily upon the endocrine system.

Rosenthal (1914) in a penetrating discussion of his results, was one of the first to compare the carbohydrate metabolism of experimental diphtheritic toxaemia with the then recognized effects of suprarenalectomy. He used massive doses of toxin, administered intravenously, and the most striking results were hypoglycaemia, circulatory collapse, and the almost complete absence of liver glycogen. (He also concluded that, since liver glycogen disappearance was associated with hypo- and not with hyperglycaemia, it is probable that carbohydrate formation was diminished.) Hypoglycaemia, low blood pressure, and a liver poor in glycogen are of course features of acute suprarenal deficiency, and so far the parallel holds good. But Rosenthal used overwhelming doses of toxin. With smaller doses, and in the earlier stages of toxaemia, the fasting blood sugar is normal or slightly raised (Schwenkter and Noel, 1930; Lawrence and Buckley, 1927; Soskin and Mirsky, 1935); it is only after massive doses, or in the later stages of toxaemia brought about by more moderate doses, that hypoglycaemia occurs. But abnormal sugar tolerance tests and insulin resistance are regularly observed at all periods, and in early toxaemia the liver glycogen may be low (Corkill and Ochoa, 1934; Cross and Holmes, 1937).

After suprarenalectomy, as in early toxaemia, glycogen can be deposited after glucose administration (Deuel, Hallman, Murray and Samuels, 1937) and both toxaemia (Corkill and Ochoa, 1934; Cross and Holmes, 1939; Dawson and Holmes, 1939) and suprarenalectomy (Britton and Silvette, 1934; Buell, Anderson and Strauss, 1936) interfere with the deposition of glycogen after the administration of lactate.

A constant finding of those who have worked on toxaemia has been that both human patients and animals rendered toxaemic experimentally are resistant to insulin. There is, on the other hand, no reason to believe that suprarenalectomy increases insulin resistance—rather the reverse, for subsequent suprarenalectomy diminishes the glycosuria and hyperglycaemia which follow pancreatectomy (Barnes, Scott, Ferrill and Rogoff, 1933).

Bamberger, Neuer and Oelkers (1938) showed once again decreased tolerance to glucose and galactose in diphtheria patients. They also published some not very satisfactory figures for the glycogen content of heart, liver, and skeletal muscle in diphtheritic toxaemia in animals.

Like Harding (1921) they stress the analogy between this condition, Addison's disease, and suprarenalectomy.[2]

Thaddea (1935) published observations on a case of Addison's disease. He was struck by the fact that infections greatly exacerbated the condition, precipitating crises which could be surmounted by the intramuscular administration of cortical extract. During these crises there was hypoglycaemia. The blood sugar was restored to normal by the administration of sufficient extract.[3]

He discusses the relationship between toxic conditions and suprarenal cortical insufficiency. He claims to have greatly prolonged the lives of guinea pigs injected with diphtheria toxin by treating them simultaneously with cortical extract and vitamin C. He has discussed this view further (1936a, b). (The alleged effect of vitamin C will be discussed separately.)

The evidence that adrenal cortical extracts exert a protective or curative effect in infective states is fairly strong. Scott and Bradford (1930) showed that adrenalectomized rats were more susceptible than normals to injections of killed B. typhosus cultures. The thymus hypertrophied in these animals after adrenalectomy.

Perla and Marmorsten-Gottesman (1930a) showed that the resistance of adrenalectomized rats to injections of typhoid vaccine and to histamine (1931b) was increased by cortical extract. Hartmann and Scott (1930) report similar results so far as typhoid vaccine is concerned.

Zwemer and Jungeblut (1935) found that guinea pigs which had received cortical extract showed cutaneous lesions of less severity than the controls after the intracutaneous injection of 1/500 to 1/50 M.L.D. of diphtheria toxin. If the extract was mixed with a lethal dose of toxin, and the mixture injected, a greater proportion survived of the animals so treated than of the control group receiving toxin alone. When extract and toxin were injected separately, a less marked protec-

[2] They also find a marked increase both in the oxygen consumption and the anaerobic glycolysis of the suprarenal cortex of diphtheritic animals as compared to that of normals. The measurements were made by shaking slices of the organ in a Warburg apparatus.

[3] Thaddea investigated other aspects of this patient's metabolism, finding a drop in total blood cholesterol, almost entirely accounted for by a decrease in the ester fraction, a rise in blood non-protein nitrogen and blood calcium and a fall in serum chloride. There was a low output of urine and of NaCl, and a high output of creatin. There were signs of renal damage (albumen and casts in the urine). The blood pressure was, of course, abnormally low, and the rise after adrenalin injection unusually small.

tive effect was observed. Administration of massive doses of cortical extract ("Eucortone") to rabbits injected with diphtheria toxin only partly restores their power to deposit liver glycogen after the injection of lactate (Dawson and Holmes, 1939).

There is reason to believe the suprarenal damage or destruction exerts a definite effect on striated muscle. Muscular weakness is a prominent feature of Addison's disease. Ingle (1936) claims that adrenalectomy in the rat diminishes the amount of muscular work which the animal is able to perform. Buell, Strauss and Andrus (1932) claim that autolysing cat's skeletal muscle forms less lactic acid than normal if the animals have been previously adrenalectomized and are suffering severe adrenal deficiency. But Corkill and Ochoa believed that toxaemia did not affect the power of muscle extract to produce lactic acid from glycogen or hexose diphosphate. Diphtheria patients do not appear to suffer from more marked muscular weakness than do those who are as gravely ill from other diseases.

It is well known that the suprarenal cortex is very rich in vitamin C. The organ undergoes change in scurvy, and a marked decrease in its vitamin C content has been recorded in various infective conditions, including experimental diphtheritic toxaemia (Harde and Benjamin, 1935; Lyman and King, 1936; Harris, Passmore and Pagel, 1937). From time to time claims have been advanced that it exerts a protective or curative effect in toxaemia, particularly in experimental diphtheritic toxaemia (e.g., Thaddea, above). Unfortunately, some of this work appears to have been carried out in a most uncritical manner, and the results carry very little conviction. Thaddea's (1935) claims have already been mentioned. Lotze and Thaddea (1937) state that vitamin C, glutathione, and cysteine will prolong the survival time of guinea pigs into which diphtheria toxin has been injected, and that it will also postpone the appearance of histologically demonstrable lesions in the livers, spleens, and suprarenals of such animals. Their published results are very unconvincing. Jungeblut and Zwemer (1935) claim that exposure of diphtheria toxin to ascorbic acid (vitamin C) in vitro destroys its toxicity (but see Sigal and King, below). Injections of ascorbic acid into guinea pigs simultaneously with diphtheria toxin results in the survival of about half the animals, the dose of toxin used normally causing 100 per cent mortality. Previous injection with ascorbic acid diminishes the severity of local reaction which follows the intracutaneous injection of sub-lethal doses of toxin. Jeney, Gagyi and Baranyai (1936) claim that guinea pigs poisoned with diphtheria

toxin live longer on a vitamin C rich diet than on a scorbutic one. King and Menten (1935) found that guinea pigs maintained at a low level of vitamin C intake were more susceptible to sub-lethal doses of diphtheria toxin than were animals receiving normal or large amounts of vitamin. Such animals, too, showed more severe local reactions. Hanzlik and Terada (1936) claim that vitamin C partially protects pigeons from diphtheria toxin and Sigal and King (1937a) claim that the glucose tolerance curves of guinea pigs on a low vitamin C intake which have been injected with diphtheria toxin are more abnormal than those of guinea pigs receiving toxin but on a higher vitamin C intake. None of these workers record anything like 100 per cent protection by vitamin C against 1 M.L.D. of toxin, no matter how much vitamin was administered. One gains the impression that the effect is certainly non-specific, and is probably not very important.

Torrance (1937) has shown that while vitamin C reacts reversibly with diphtheria toxin in vitro, there is no relationship between the potency of the toxin and its effect on the vitamin; that heated and therefore inactivated toxin still reacts with the vitamin, and finally that toxin which has reacted with the vitamin retains its potency. Sigal and King (1937b) show that vitamin C has no action on diphtheria toxin in vitro if it is neutralized and buffered before being mixed with the toxin. This obviously throws doubt on the experiments mentioned above which were adduced in evidence that vitamin C inactivated toxin "in vitro". Zilva (1937) categorically denies that vitamin C protects guinea pigs against diphtheria toxin. Herbrand (1935), Messer (1936) and Bernhardt (1936) support Thaddea's contention (above, p. 449) that the simultaneous administration of vitamin C and suprarenal cortical hormone exert a protective effect in experimental and clinical diphtheritic toxaemia, though neither alone is effective. Since both Messer and Bernhardt gave other forms of treatment, including serum, along with the cortical extract and vitamin C, their evidence is valueless. Dawson and Holmes (1939) find that early (24 hrs.) diphtheritic toxaemia very much retards the rate at which lactate disappears from the blood stream after it has been injected intravenously: if massive doses of suprarenal cortical extract are injected, the rate at which lactate disappears is increased, but is not restored to normal.

It will be seen from the above discussion of the literature that there is a considerable body of opinion which seeks to refer some of the important metabolic changes occurring in toxaemia to lesions of the suprarenal cortex. That such damage may account for some of the phe-

nomena observed is highly probable, but that it is the most important cause of the metabolic disturbances does not seem, to the writer at least, to have been established.

Before the functions of the suprarenal cortex were at all understood, attention was focussed on the suprarenal medulla, and the pioneer workers in this field (e.g., Rosenthal, 1914; Cramer, 1926; Lawrence and Buckley, 1927; Evans and Zweckwer, 1927) were disposed to consider that some or all of the changes in toxaemia were to be attributed to hypersecretion, perhaps later followed by hyposecretion, of the adrenal medulla and possibly of the thyroid. Giganticism and acromegaly are well recognized results of hypersecretion of an endocrine gland which are sometimes caused by infective processes, and apart from this there is a formidable body of experimental evidence pointing to the importance of the suprarenal medulla in toxaemic conditions.

One of the most constant findings has been that toxaemia causes resistance to the hypoglycaemic action of insulin, and a "diabetic" type of glucose tolerance curve. Lawrence and Buckley (1927) and Corkill (1932) found that the insulin resistance of toxaemic rabbits was abolished by ergotamine tartrate. The glucose tolerance curves of some of the patients studied by Elkeles and Heiman (1927) were restored to normal by the same substance. Evans and Zweckwer (1927) found that the hyperglycaemia following injection of killed bacterial cultures into rabbits was abolished by section of the splanchnics, and Mikami (1925) reports similarly on the hyperglycaemia which he observed after the injection of diphtheria toxin. It is now recognized that adrenalin may have a twofold effect upon liver glycogen: on the one hand it causes glycogenolysis, while on the other, by increasing the blood lactate, it subsequently causes an increased deposition of liver glycogen. It is believed (see discussion by Marks (1936)) that the "recovery phase" of the blood sugar which follows a fall produced by insulin is caused by an outpouring of adrenalin, and Barnes, Dix and Rogoff (1933) have shown that the denervation of the suprarenals and destruction of their medullae renders dogs "insulin sensitive."

Cross and Holmes (1937) demonstrated the synthesis of glycogen by slices of rabbit's liver "in vitro." They found that the phenomenon did not occur when the liver slices were taken from a rabbit injected 24 hours previously with diphtheria toxin, and they found also that glycogen synthesis was inhibited when a very small quantity of adrenalin was added to the fluid in which slices of the livers of normal rabbits were suspended. Kotliarov (1938) suggests that extracts of the livers

of cats from which the suprarenal medullae have been removed have less glycogenolytic power than extracts from the livers of normal cats. So far, therefore, as resistance to insulin, absence of liver glycogen, and perhaps the abnormal glucose tolerance are concerned, the facts seem to fit with the supposition that the adrenal medullae are over-active in toxaemia. But again we are confronted with other observations which make it extremely unlikely that such a hypothesis can afford a complete explanation of the facts. If we are to account for the phenomena by assuming an increased secretion of adrenalin we must expect hyperglycaemia and a raised blood pressure regularly to occur. A very great many observations have been made by different workers upon the fasting blood sugar level of human patients and animals in diphtheritic and other toxaemias. There is no evidence that, in early toxaemia, the fasting blood sugar level is consistently and markedly raised, though most workers record a few cases in which this was so. In severe toxaemia, on the other hand, hypoglycaemia is very consistently observed, and animals often die in hypoglycaemic convulsions. Only in some of Lawrence and Buckley's and Schwenkter and Noel's rabbits, which died in 5 to 7 days (most workers have used doses of toxin lethal in 72 hours or less) was hyperglycaemia often observed. The first named workers record hyperthermia and intense hyperglycaemia as an occasional terminal phenomenon, and the last named noted that animals which survived for more than 72 hours often died in coma, while those dying after shorter periods usually died in convulsions, presumably hypoglycaemic in nature. By contrast the injection of some kinds of living (Williams and Dick, 1932) and dead (Zweckwer and Goodell, 1925a) organisms regularly gave rise to hyperglycaemia, and anaphylactic shock may be accompanied by extremely high blood sugars (Zweckwer and Goodell, 1925b). Thus, if we are to explain the regularly occurring insulin resistance, abnormal glucose tolerance curves, and low liver glycogen values solely by an increase in the activity of the suprarenal medulla, then we must expect to find a regularly occurring and marked hyperglycaemia—which is conspicuous by its absence. Similarly, we should expect an increased blood pressure, whereas low blood pressure is a most striking feature of experimental diphtheritic toxaemia. While therefore there is strong evidence that over-activity of the suprarenal medulla may account for some of the important metabolic disturbances in toxaemia, it is at present difficult to believe that it can account for the others, probably equally important. Edmunds and Johnston (1928) and Myers (1933) have observed that

in the late stages of experimental diphtheritic toxaemia adrenalin ceases to have its accustomed effects on the organs innervated by the sympathetic system. Myers found that the adrenal glands of the toxaemic animals still contained appreciable quantities of adrenalin[4], for extracts made from them would produce the usual effects upon isolated or perfused organs, and other preparations from normal animals. In spite of this, the heart, intestines, etc. of the toxaemic animals quite failed to respond either to extracts of fresh adrenals, or to commercial preparations of adrenalin. Pituitrin and nicotine also failed to bring about their usual effects on the heart and intestine, and digitalis added to the isolated perfused toxaemic heart produced its usual "toxic" effect, which was not, however, preceded by a phase in which the amplitude of the beat was increased as it is in the normal heart. These findings suggest that diphtheria toxin exerts a profound effect upon the tissues, as well as upon the endocrine glands.

Recent work on the anterior pituitary has emphasized so strongly its rôle as a regulator of carbohydrate metabolism that the possibility that it plays its part in the phenomena of toxaemia cannot be ignored.

Cope and Marks (1934) found that the injection into rabbits of crude anterior pituitary extracts produced in these animals insensitivity to the hypoglycaemic action of insulin. Young has published a series of papers showing that this substance, which is not capable of a general "diabetogenic" action, can be separated from a number of other physiologically active fractions of the anterior pituitary. He has named it the "glycotropic factor" (Young, 1938b; other references in the paper cited). The action of this factor perhaps furnishes a counterpart to the insulin sensitivity occurring after hypophysectomy.

Himsworth and Scott (1938), repeating the observations of Mann and Bollman (1935) that insulin accelerates the fall of the blood sugar which occurs in a hepatectomized animal, find that this acceleration is inhibited in animals which, before hepatectomy, had received an injection of the "glycotropic" hormone; although the rate of the fall of the blood sugar which regularly occurs in the absence of injected insulin is in no way affected.

Himsworth has developed the view that diet is an important factor in determining both the sensitivity of normal human beings and animals (rabbits) to insulin, and also the form of the glucose tolerance curve. Diets high in carbohydrate increase insulin sensitivity and glucose

[4] C. A. Ashford (personal communication) has confirmed this.

tolerance ("Straub-Traugott phenomenon"); diets low in carbohydrate cause insulin resistance and lower glucose tolerance (Himsworth, 1934) (for summary of work with normal and diabetic patients see Himsworth, 1932, 1936).

It is thus possible that diet may modify insulin sensitivity and glucose tolerance by an effect upon the anterior pituitary. So far as the writer is aware, there is so far no direct clinical or experimental evidence to connect these phenomena with the insulin resistance and decreased glucose tolerance of toxaemia; yet histological changes in the anterior pituitary have been reported as occurring in such conditions. Both human beings and animals suffering from severe toxaemias are likely to refuse food. Starvation decreases glucose tolerance even in the normal organism. It is well recognized not only that the occurrence of infection markedly increases the insulin requirements of diabetics, but that, apart from infections, certain diabetics belong to an "insulin resistant" group. Such cases have been investigated by Himsworth (1936; and other publications to which references will be found in that cited) who draws a parallel between their case and that of patients rendered "resistant" or "insensitive" to insulin by a carbohydrate poor diet. The fact that a diabetic-like condition (Young, 1938b) can be induced in animals by injections of anterior pituitary extract (a counterpart to the amelioration of the symptoms of pancreatectomy brought about by removal of the pituitary) makes it almost impossible, apart from the work already mentioned, not to seek for a connection between insulin "resistance" brought about by diet, and the activity of the anterior pituitary; and also to explore the possible importance of lesions of the anterior pituitary in the alterations of carbohydrate metabolism seen in some, at least, of the phases of toxaemia.

It will be obvious from our discusson of the effects of the endocrine glands that it is in the highest degree probable that toxaemia may act primarily upon them, and thereby modify metabolic processes. Important, however, as the endocrine glands may be as regulators of metabolic processes, the fact that animals deprived of pancreas, and suprarenals, or pancreas and pituitary will survive for some time makes it clear that the bodily functions may be maintained without either stimulation or control by these glands. Moreover, the actual work of metabolism is carried on by tissues such as the liver, kidneys, brain and skeletal muscles. In toxaemia these organs often suffer damage which can readily be detected by histological methods, and biochemical, no less than structural lesions may reasonably be expected. Whether the

anterior pituitary, thyroid, adrenals and pancreas are damaged or not, the liver cannot be expected to store and liberate glycogen, to carry out the work of gluconeogenesis, or to deaminate amino acids and form urea in a normal manner if its enzyme systems are deranged by toxaemia. In cases of severe liver damage hypoglycaemia is known to occur, often as a fatal event, and there seems no need to attribute it to any cause other than a failure of liver function, and in particular of the power of gluconeogenesis.

Yannet and Darrow (1933a, b) have approached the question of diphtheritic toxaemia from this point of view. In their first paper, they report the effect of diphtheritic toxaemia upon the blood sugar, blood non-protein nitrogen, blood amino nitrogen, and blood lactic acid. Their results, so far as blood sugar was concerned, conformed to those of other workers. With large doses of toxin, there was either a small rise, no change or a slight fall at the end of 24 hours, followed by hypoglycaemia after 48 hours. With smaller doses of toxin there was hyperglycaemia. The blood non-protein nitrogen rose in all cases; in a few of the less severe cases, the blood amino nitrogen fell again, but in the remainder it rose. The blood lactic acid showed a steady rise in all cases.

In their second paper, they studied the effect of diphtheritic toxaemia on the power of the liver, cardiac muscle, and skeletal muscle to synthesise glycogen from glucose, with and without insulin. So far as initial values were concerned they observed that while toxaemia very much depleted the glycogen content of the liver, it hardly affected that of skeletal and cardiac muscle. The power of the liver in toxaemia to lay down glycogen in response to injections of glucose was much less than in the normal animal (cf. however, Corkill and Ochoa; Cross and Holmes) and this was little affected by the simultaneous injection of insulin. On the other hand, in toxaemia the heart muscle deposited rather more glycogen in response to injections of glucose, and distinctly more in response to injections of glucose and insulin, than did normal heart muscle. The authors interpret these results as indicating a failure in the glycogenetic power of the liver.

The development of the technique for studying the metabolism of slices of tissue suspended in Ringer's solution or other medium, to which various substrates, drugs, etc., may be added at will, has opened up a field of investigation which promises to be useful in the study of the effects of toxaemia upon individual organs. It is obvious that those who employ such methods must be on guard against errors in interpre-

tation; but so far similar methods employed in work on normal tissues have met with marked success, and again and again have confirmed and extended the conclusions reached in studies on the whole animal by the classical methods of biochemistry. The use of the isolated tissue slice in the study of the metabolism in toxaemia obviously permits metabolic studies to be correlated closely with histological examination. The metabolic behaviour of slices of an organ can be observed, and a portion of the same organ used for microscopical section. That metabolic studies on isolated slices will permit the changes observed to be ascribed to the effects of the toxin directly upon the organ studied rather than indirectly through the endocrine system is probable, but not certain. During the course of the experiment, no hormone can reach the tissue unless it is deliberately added by the experimenter, but clearly, unless the tissue is from an animal from which the endocrine gland under consideration has been removed some time previously, it may have carried with it a supply of the substance sufficient to last during the period of the experiment. This possibility can be explored, but it will require time to do so. In the meantime, it is readily possible to demonstrate that marked differences exist between the metabolism of isolated liver tissue from normal and from toxaemic animals. The power of liver to form carbohydrate from non-carbohydrate substances (gluconeogenesis) has been often demonstrated in the whole animal and in the isolated perfused liver. That it is possessed by isolated slices of liver was shown by Gemmill and Holmes (1935), Bach and Holmes (1937) and Cori and Shine (1936); and that it is damaged in diphtheritic toxaemia was shown by Cross and Holmes (1937). They found that after toxaemia of 48 hours' duration in rabbits which had received a dose of toxin lethal in 56 to 72 hours, the power of the isolated liver slices to form fermentable carbohydrate from lactic acid, pyruvic acid, and alanine was diminished by 70 per cent or more. The power to form fermentable carbohydrate from glycerol, glycerophosphate, glyceric aldehyde, and butyrate, was also diminished, but to a lesser degree. After only 24 hours' toxaemia, the diminution of gluconeogenetic power from any of these substrates was very much less marked.

Since Corkill and Ochoa had found that glycogen was no longer deposited after an injection of lactate into a 24 hour toxaemic animal, Cross and Holmes tried, with success, to demonstrate glycogen formation in liver slices. The synthesis from lactate and pyruvate was small, that from glucose and still more from fructose, considerably larger. Toxaemia of 24 hours' duration, though it had already been

found to have little effect upon gluconeogenesis, almost completely prevented the formation of glycogen in the slices from lactate or pyruvate, though some glycogen was still formed from glucose. Thus, in *early* toxaemia, it is the power to deposit glycogen rather than to form carbohydrate which the liver seems to lack. If this is so, it follows that, in early toxaemia, an injection of some substance such as lactate which, in the normal animal, is stored in the liver as glycogen, and raises the blood sugar only temporarily or not at all, should give rise to hyperglycaemia. This has been found to be indeed the case (Dawson and Holmes, 1939). As was mentioned earlier, Cross and Holmes also found that addition of a very small quantity of adrenalin to the liver slices inhibited glycogen formation (though not the formation of glucose). It is tempting, therefore, to ascribe the effect to the presence of excess of adrenalin. But in these experiments, glycogen formation from glucose was inhibited by adrenalin as much as was that from lactate, and though such an explanation is in line with some of the earlier work, the objections previously raised against that view remain. It seems probable that toxaemia somehow increases the rate of breakdown of glycogen, rather than that it inhibits its formation, for otherwise it is difficult to account for the fact that in the 24 hour toxaemic animal some glycogen is still formed from injected glucose, but none from injected lactate. If, however, the reversible reaction glucose $\rightleftarrows$ glycogen is shifted to the left in toxaemia, the presence of a high concentration of glucose following glucose injection might still suffice to shift it to the right.

It has become clear from later results (Dawson and Holmes, 1939) that changes besides failure to deposit glycogen take place even after 24 hours' toxaemia, for there is a very marked slowing (as compared with the normal) in the rate at which lactate disappears from the blood stream after it has been injected intravenously, and a large decrease in the rate at which liver slices from toxaemic rabbits, as compared with normal ones, cause lactate to disappear. Yannett and Darrow (1933b) observed a progressive rise in blood lactate during toxaemia. Cross and Holmes (1937), Cross (1938) have compared the oxygen consumption, respiratory quotient, and change in acid-base equilibrium (sometimes known as "glycolysis") of slices of liver from normal, 24 hour toxaemic and 48 hour toxaemic rabbits. As toxaemia increases, the oxygen consumption and respiratory quotients fall. If no substrate is added to the tissue slices, about the same amount of acid is produced after 24 hours' toxaemia as in the normal tissue, but after 48 hours the

acid production is decreased. Lactate and pyruvate added to normal tissue markedly increase the oxygen consumption and raise the R.Q., and acid radicles are removed. It has already been shown that some of it is converted into carbohydrate, and the increased oxygen consumption and raised respiratory quotient show that some of it is burned. In tissue from 24 hour, and still more from 48 hour toxaemic rabbits, the oxygen consumption is decreased, the R.Q. is lowered, and, in the presence of lactate or pyruvate, much less acid disappears than is the case with tissue from normal animals. These observations on isolated tissue are thus in harmony with those on the whole animal, and they indicate that even after 24 hours' toxaemia, still more after 48 hours, there is a disturbance of the oxidation mechanisms as well as of the power to deposit glycogen. It is interesting that the power to form carbohydrate is maintained in 24 hour toxaemia, and is markedly affected only after 48 hours, in spite of the fact that at the earlier stage the oxidation mechanisms are perceptibly damaged, and the rate of disappearance of lactate added to isolated tissue is decreased, as is the rate of disappearance of injected lactate. A further point of interest is that the damaging effect of toxaemia is greater on carbohydrate synthesis from some substrates than from others. Lactate, alanine, and pyruvate form little carbohydrate in the isolated liver after 48 hours' toxaemia, while glycerol, glycerophosphate and glyceric aldehyde still form a good deal (Cross and Holmes, 1937). The oxygen consumption, respiratory quotient, and acid production are only a little less after 48 hours' toxaemia than in normal tissue when glycerol is added as a substrate.[5]

Work of this kind at present involves investigation of the normal mechanisms as well as the disturbances which may occur in toxaemia, and the interpretation of results is often uncertain because the nature of the normal machinery is not sufficiently understood. For instance, the disappearance of lactate (Dawson and Holmes, 1939) and of pyruvate (Cross, 1938) when shaken with isolated liver slices from normal animals is much greater than the disappearance of acid measured

[5] A report has just come to hand of the work of Lyman and Barron (Proc. Am. Soc. Biol. Chemists, p. lxiii, Toronto, 1939). These workers found that the oxidation of pyruvic acid, lactic acid, alanine, aspartic and succinic acids, and choline by the isolated kidney tissue of rats poisoned by diethylene glycol was much diminished when compared with their oxidation by slices of normal rats' kidneys. Ammonia production was also reduced. It was thought that the dehydrogenating enzymes were most affected. Clinically, the animals suffered from a severe nephritis.

manometrically. This means, of course, that lactic and pyruvate acids are converted, during the experiment, into some other acid or acids which have not so far been identified.

Most of the work so far discussed has been concerned with the effects of diphtheria toxin, both in human cases and in animals. That this should have been so is natural, for on the one hand the disease is of great clinical importance, and on the other diphtheria toxin can easily be obtained and standardized, is stable, and has easily reproducible effects when injected into animals; effects, that is to say, which are reproducible within the limits of variation which naturally occur from one animal to another. Making use of an isolated enzyme system, and of toxins from bacteria of a different type, Wooldridge and Higginbotham (1938) have investigated the effect of certain anaerobic organisms—Cl. Welchii, Cl. Oedematiens, Cl. Septique, Cl. Tetani—upon the enzyme system responsible for the oxidation of succinate by extracts of the finely ground small intestines of rats. Positive results were obtained with Cl. Welchii type A., and the authors thought that the "α" toxin was probably responsible. The oxygen uptake of the preparation was definitely inhibited, and the inhibitory effect was reversed both by heating the toxin and by the addition of the appropriate antiserum. Since neither the dehydrogenase nor the indophenol oxidase enzymes appeared to be affected, the authors thought that the toxin probably acted upon some carrier component of the system.

Extremely interesting results have, however, been obtained by the use of non-bacterial toxic agents. Minot (1926) in the course of a study of the effect of carbon tetrachloride administered to dogs found that the substance was particularly lethal to animals kept on a diet low in calcium. High calcium diets exerted considerable protection, and calcium administration relieved the condition. Apparently, the administration did not have much effect on the liver damage, as judged by the histological picture, but did nevertheless save the animals' lives.

Minot and Cutler (1929a, b) found that guanidine, or a guanidine-like substance accumulated in the blood stream of animals suffering from carbon tetrachloride or chloroform poisoning, and of patients with eclampsia or pre-eclamptic toxaemia. Carbon tetrachloride and chloroform produce severe "central necrosis" of the liver, and liver lesions are found in eclampsia (Stander, 1929). Minot and Cutler observed that both experimental animals and patients suffered from hypoglycaemia, and Titus, Dodds and Willetts (1927) record the occurrence of hypoglycaemia in eclampsia. The hypoglycaemia occurring

in animals could be relieved by glucose injections, but calcium administration seemed also to be essential, nor were the nervous symptoms relieved by administration of glucose without calcium. The administration of guanidine (Minot and Cutler, 1929b, c) causes symptoms very like those caused by carbon tetrachloride, which can be relieved by calcium administration. Guanidine retention and calcium reserve are said to act as antagonistic factors in carbon tetrachloride and chloroform poisoning.

Minot (1931) showed that there was a great increase of lactic acid in the blood of dogs poisoned either with carbon tetrachloride or guanidine. In rats, doses of 50 to 75 mgm. of guanidine chloride per 100 grams of body weight caused a fall in blood sugar, a rise in blood lactic acid, a fall in liver glycogen, and a decrease (less marked than in the liver, however) of the total glycogen of the body. These effects were, to a great degree, prevented by calcium administration. In dogs poisoned with carbon tetrachloride, the calcium effect was in turn nullified by the administration of ergotamine tartrate. Minot, Dodd and Saunders (1934) gave repeated doses of 100 mgm. of guanidine hydrochloride subcutaneously to dogs. They found that it gave rise to a severe acidosis, which terminated fatally. The animals could not utilize lactic acid, which accumulated in the blood and was excreted in the urine. There was a fall in the blood CO_2 and bicarbonate. The administration of bicarbonate exacerbated the condition, perhaps by increasing the guanidine concentration in the blood, or by decreasing the ionic calcium. The condition was improved if calcium was given with the alkali.

Further work by Minot and her collaborators has served to emphasize the fact that increases in blood guanidine are often found in toxaemic conditions in which there is serious loss of fluid, such as acute gastroenteritis in infants, and that conversely, guanidine poisoning is associated with dehydration and finally with circulatory collapse (Minot and Dodd, 1933; Minot and Keller, 1936). This action of guanidine on the circulation is counteracted by calcium, and is thought to involve the autonomic system (Minot, 1938a, b).

Minot and Dodd (1933) discuss the toxicology of guanidine, and point out that there is reason to believe that guanidine is liberated during necrosis of tissues.

Marshall and Rowntree (1915) found that there was decreased tolerance to galactose and laevulose in dogs poisoned with phosphorus or chloroform. Althausen and Thoenes (1932a, b) investigated the effects

of phosphorus and chloroform poisoning upon the carbohydrate metabolism of rabbits. Their experiments were rather few in view of the variable behaviour of the animals and the complicated nature of the results. Rabbits were given small doses of phosphorus in oil, injected subcutaneously every day. Their livers underwent fatty degeneration, but if the dose was omitted for a day or two, marked cellular regeneration occurred and a notable degree of resistance was established. Modified glucose tolerance tests were performed. The tests consisted in giving 5 grams of glucose in 100 cc. of water by stomach tube, together with the subcutaneous injection of 1 unit of insulin. These tests were begun 4 days after the first phosphorus injection, and continued daily, the animals feeding well for most of the time.

The administration of the phosphorus caused a diminution of glucose tolerance; in the later stages of the poisoning there was a tendency to marked hypoglycaemia at the end of the test. The hyperglycaemia normally evoked by adrenalin was less than usual. The administration of lactate (2 gm/kilo by stomach tube) caused a hyperglycaemia. After 4 days on phosphorus, adrenalin failed to give a hyperglycaemia, and the liver glycogen was reduced. Glucose and insulin failed to raise the liver glycogen.

Chloroform poisoning (Althausen and Thoenes, 1932b) caused by a single dose of 1 cc. of chloroform given subcutaneously in 33 per cent solution in oil gave rise, in the acute stage, to some hypoglycaemia. There was diminished tolerance to dextrose and insulin, and the hyperglycaemia which normally follows adrenalin injection was diminished, and was sometimes replaced by a hypoglycaemia. During the recovery stage glucose and insulin, or even glucose alone sometimes gave a marked *hypoglycaemia*. The liver glycogen was said to be reduced, but no proper control figures were published.

These experiments are difficult to interpret: the authors were of the opinion that they were dealing with liver damage uncomplicated by other lesions, and it is possible that their results were caused by alterations in the gluconeogenetic and "homoeostatic" mechanisms of the liver. They quote Williamson and Mann (1923), Opie and Alford (1915), and Mosiman and Whipple (1912) as supporting the view that chloroform poisoning causes damage only to the liver. This is quite at variance with what is commonly taught on the subject, and perusal of the papers quoted shows that, while Mosiman and Whipple did express such an opinion, they did not give (in that paper) any experimental evidence for it. Opie and Alford dealt with the effects of diet in liver

lesions caused by phosphorus, chloroform and alcohol and did not investigate any organ other than the liver, while Williamson and Mann are at pains to make it very clear that they do *not* ascribe the death of the majority of their animals after chloroform or phosphorus to liver damage, since only in a few cases was there a hypoglycaemia, rise in blood uric acid, or the appearance of symptoms at all like those following hepatectomy. On the contrary, they insist that death in many cases must have been due to damage to other organs, such as the heart or kidneys.

It is difficult to understand why these authors were so misquoted, nor is it easy to understand why Althausen and Thoenes complicated their experiments by giving insulin as well as glucose and at the same time failed to use more animals or to do a sufficient number of control experiments on the change of liver glycogen.

A very interesting paper on the effects of phosphorus poisoning on carbohydrate metabolism is that of Frank and Isaac (1911). In spite of the fact that they had to use "macro" methods for blood sugar determinations, these authors showed that in rabbits which had received a dose of phosphorus fatal in about 4 days, there was usually a terminal hypoglycaemia. Glucose tolerance tests showed a grossly impaired tolerance, and, again in the later stages, adrenalin failed to give rise to a hyperglycaemia. The authors argue that, in the earlier stages of poisoning, there is a failure on the part of the liver to deposit or retain glycogen. Later, there is a failure of gluconeogenesis, and particularly, they think, in the conversion of lactate to carbohydrate. While they did not bring very much experimental evidence to support these views, they seem, in fact, to fit very well with later findings discussed earlier in this review.

Neubauer (1909) had earlier claimed that after the subcutaneous administration of optically inactive sodium lactate to normal rabbits, there was an increased excretion of other soluble acid in the urine, part only of which was lactic acid. This phenomenon was little affected by doses of phosphorus fatal in about four days. This does not seem to fit very well with the conception of failure in the conversion of lactic acid to carbohydrate as an important feature of phosphorus poisoning.

It seems possible that the discrepancy between the findings of Frank and Isaac and those of Williamson and Mann are to be explained by the fact that the latter authors probably used smaller doses of phosphorus than the former. In their animals the brunt of the effect may have fallen upon organs other than the liver, lesions which were

responsible for the death of the animals, while Frank and Isaac used amounts of phosphorus which quickly caused extensive and fatal liver damage, the effects of which masked those of lesions in other organs.

There is an extensive literature on the effect of phosphorus poisoning upon protein metabolism. To enter into it here would lengthen this review very considerably, and it has been decided, perhaps rather arbitrarily, not to do so. A brief discussion, with many references to the literature, will be found in Lusk's *Science of Nutrition* (1928) and in Marshall and Rowntree's (1915) paper; Frank and Isaac (1911) also refer to a number of the earlier papers.

Hartmann and Senn (1932c) have developed a technique of lactate administration for relief of acidosis in patients (1932a, b). They found that after intravenous (racemic) lactate administration to a group of four patients with catarrhal jaundice ("acute hepatitis") there was a slight but definite delay in the complete metabolism of lactate, as judged by measurement of blood lactate and the CO_2 content of the blood, and a definite hyperglycaemia which, however, disappeared within two hours.

A very similar technique, in which, however, intravenous doses of 50 to 75 mgm. of sodium d-lactate per kilo of body weight were given was used by Stoffer, Dantes, Newburger and Sobotka (1937a). In normal patients, d-lactate injections of this magnitude gave rise to an elevation of blood lactic acid which reached its peak in 5 minutes, and had disappeared within 30 minutes, there was no change in blood CO_2 or blood sugar, and no lactic acid appeared in the urine. They found (1937b) a higher peak in blood lactic acid, and a delayed return to normal in 3 cases of catarrhal jaundice, 4 cases of arsphenamine poisoning, and 1 case of cirrhosis of the liver with superimposed necrosis.

Stoffer, Dantes and Sobotka (1938) applied this test to a group of patients with jaundice with hepatitis, and to another group with jaundice due to extra-hepatic causes. There was delay in return of the blood lactate curve to normal in the former group, but not in the latter. A small group of diabetic patients were also investigated. One only, who had an enlarged liver, showed a delayed curve; but delay in the return of the blood lactate to normal was observed in 3 out of 6 cases of muscular disease.

Stoffer, Dantes and Sobotka (1937) found lesions of the liver and kidneys in dogs with acute arsphenamine poisoning. They state that the other organs were normal, but do not mention any special examination of the endocrine organs. They observed a diuresis, increase in

blood non-protein nitrogen, inorganic phosphate, and lactic acid and a fall in the CO_2 and chloride content of the blood. Three cases showed severe hypoglycaemia. The urinary lactic acid was increased and the urinary chloride and inorganic phosphate diminished; there was albuminuria.

It will be seen from the above discussion that there is a definite parallelism between the changes in carbohydrate metabolism brought about by diphtheria toxaemia on the one hand, and by poisoning by carbon tetrachloride, chloroform, phosphorus, organic arsenicals, guanidine, and apparently in some cases by the toxaemia of eclampsia, on the other. The common features are liver damage, decreased glucose tolerance, low liver glycogen, hypoglycaemia, inability to remove lactic acid from the blood stream; these phenomena may all reflect a diminished power of gluconeogenesis. Cross and Holmes observed (unpublished experiments) that guanidine added to slices in vitro could abolish their power to form carbohydrate. How far, however, the changes all spring from a common cause, only further work will show. Clinically, the conditions just mentioned may be associated with jaundice, although obvious jaundice is certainly not seen in diphtheria occurring either clinically or in experimental animals. So far as we know, no observations have yet been made either on the occurrence of guanidine in the blood in diphtheria or upon the effect of calcium in that condition. The report that ergotamine tartrate prevents the beneficial effect of calcium in experimental carbon tetrachloride poisoning, if confirmed, would suggest that the calcium may act through the autonomic nervous system, perhaps on the suprarenal glands.

There have been fewer observations of the effect of toxaemia upon the metabolism of nitrogen than upon that of carbohydrate. Since it is becoming more and more obvious that, once amino acids have suffered deamination, the fate of their carbon residues becomes indistinguishable from that of three or four carbon chains derived from other sources, it may well become misleading to press the distinction between the effect of toxaemia upon the metabolism of nitrogenous bodies and of carbohydrate or fat. So far as deamination and urea formation are concerned, it is at present difficult to distinguish between the effect of toxaemia upon the liver and the kidney. Kidney damage, or even circulatory failure, may obviously cause a rise in non-protein nitrogen of the blood, by reason of diminished urea excretion; though deamination and urea formation may be normal. On the other hand, since the kidney deaminates very rapidly but contributes a hitherto

unassessed fraction to the total deaminating power of the body, accumulation of amino acids may be partly due to kidney damage as well as to liver damage.

Yannet and Darrow (1933) state that their toxaemic rabbits showed necrosis of the liver parenchyma, a glomerular and tubular nephritis, degeneration of the heart muscle fibres and haemhorrages in the suprarenals. The majority of their animals showed progressive increase both in blood non-protein nitrogen and in blood amino nitrogen. Since the blood amino nitrogen is a comparatively small fraction of the total non-protein nitrogen, there was presumably marked and progressive urea retention which might be attributed to the kidney lesions, the circulatory failure, dehydration (with consequent failure in renal function), or to all three causes combined. It has also been shown that the blood non-protein nitrogen is increased in adrenalectomized animals, so that a rise of blood non-protein nitrogen in a toxaemic animal might be caused by suprarenal damage.

Cross and Holmes (Cross, 1938) found that urea formation from slices of rabbit liver both with and without alanine appeared to be diminished by about 30 per cent of the normal value when the slices were taken from the livers of 48 hour toxaemic animals. The initial values were much higher in the toxaemic animals, and this could well be accounted for by a raised blood urea, which was observed in the cases in which it was looked for

Wakeman and Morrell (1930) found that in experimental yellow fever in monkeys there occurred marked histological lesions of the liver, and often of the kidneys. In the later stages of the disease there was usually a rise in blood non-protein nitrogen, but the amino acid nitrogen was much more increased than the urea nitrogen. This appeared to indicate damage to the deaminating power of the liver or kidneys, or both. The decreased amount of urea might have been due simply to decreased deamination or to a failure in urea synthesis. In the latter case an increase in blood ammonia might have been expected, but was not recorded. Hypoglycaemia was observed.

Marshall and Rowntree (1915) poisoned dogs with phosphorus and chloroform. The N_2 partition in the urine was always disturbed in the direction of increase in amino acid nitrogen and decrease in urea nitrogen. The authors were of the opinion that in phosphorus poisoning, at any rate, renal changes contributed less to the picture than did damage to liver function. In this condition the non-protein nitro-

gen, urea, and amino acid nitrogen was increased both in blood and urine, and this they attributed to increased protein catabolism.

The work which has been reviewed extends over a considerable period of time, and the reports of it are scattered through a number of journals. It is obvious that many of the authors were not aware of the results previously obtained by others. It is easy, however, to follow a common thread which runs through the work. It leads directly to the suggestion that in many different toxaemic conditions the damage falls most heavily upon the machinery of carbohydrate metabolism. At the same time there is thrown into sharp relief the question whether the damage is primarily to endocrine organs which control the normal speed, direction and balance of carbohydrate metabolism, or whether the essential feature is disturbance of those emzyme systems of the cells of the liver and other organs which deal with carbohydrate molecules. There is so much evidence on both sides, that the only safe conclusion at present is that probably both factors come into play in producing the picture of disturbed carbohydrate tolerance, insulin resistance, and finally failure in glyconeogenesis with which we have become familiar. Lines of investigation which should serve further to clarify the problem lie open, and the practical advantages to be gained from such a clarification are obvious, for replacement of deficient endocrine secretions is becoming almost daily more and more within the reach of practical therapeutics.

The trend of modern work on intermediary metabolism may perhaps lead to a simplification of our conception of the effect of toxaemia, for it is becoming plainer and plainer that protein, fat, and carbohydrate all are broken down, during the course of metabolism, to compounds containing three or four carbon atoms, and that once they have reached this stage the products of the main types of foodstuff become indistinguishable. There is strong evidence that it is upon the metabolism of these three and four carbon compounds that toxaemia produces its effect, so that it may well be misleading to think of the effects of toxaemia upon fat or protein metabolism as phenomena distinct from its effect upon carbohydrate metabolism or upon the action of insulin. It may, on the contrary, become both possible and profitable to consider its effect, for instance, upon urea formation or upon the oxidation of pyruvic acid as being essential features of the problem. A "normal" sugar tolerance curve is the result of many factors—an intricate balance of endocrine secretions, the state of the enzyme systems in the various

tissues and the fulness or depletion of carbohydrate stores in the liver and perhaps elsewhere. The observation that the sugar tolerance curve deviates from its normal shape merely tells us that one or more of these conditions is disturbed, and a much closer analysis is necessary before we can describe at all precisely what has happened.

Biochemical findings can with great profit be compared with histological ones. At a crude level this has already been done. For instance Cross and Holmes (Cross, 1938) found that 24 hours after the injection of diphtheria toxin into rabbits there was little to be seen in the way of histological change in sections of the liver, and that while glycogen storage was grossly disturbed, gluconeogenesis was but little affected. After 48 hours' toxaemia necrotic patches were usually obvious in the liver, and gluconeogenesis from lactate and pyruvate were generally much reduced; but in a few of the 48 hour toxaemic animals gluconeogenesis went on as in the normals, and none of these animals showed histological signs of liver damage. The correlation between histological and chemical findings was striking.

Advances in knowledge of pathological conditions must always depend ultimately on clinical observation and study, for only so can the problems be posed and defined; "clinical" here is used in a wide sense, for probably a very great deal more use could be made of veterinary material than is at present the case. But the writer would stress the absolute necessity of combining the use of animal experiment with clinical observation. Without the use of animals, material is difficult to obtain, experimental scope is limited, and adequate controls often impossible. It is true that disease in man is not necessarily—perhaps not often—exactly reproducible in animals. Much of the work here discussed has indeed to do with liver damage during diphtheritic toxaemia, whereas the prominent feature of human diphtheria is circulatory collapse rather than obvious disturbance of liver function. But it is in the highest degree unlikely that the effects of bacterial or other poisons upon fundamentally important enzyme systems will differ from species to species, and it is with such effects that we are primarily concerned. Once these problems have been unravelled, it is likely to be a relatively simple matter to say why the particular manifestations of disease differ from one species to another.

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THE PRESENT STATUS OF THE BARBITURATE PROBLEM

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The subject assigned to the writer for discussion is so wide in scope that it seems feasible to attempt only a broad survey, the primary objective being to furnish leads to various aspects of the barbiturate[1] problem as well as to supply a certain amount of useful information.[2]

Brain. The brain is the chief site of action in that hypnosis and amnesia, and in overdosage, anesthesia and coma, are the commonly expected dominant effects. Masserman (113), on the basis of electrical stimulation of the anterior hypothalamus, found that barbiturates (amytal) caused a depression or diminution of reactivity of this region, while after small doses, the motor cortex was relatively unaffected. On the other hand, Fulton and Keller (43) saw depression of the cortex after nembutal (pentobarbital) prior to depression of the hypothalamic centers and explain, on this basis, the period of excitement so often seen in laboratory animals.

On the basis of a study of regions of action of certain relatively effective analeptics, indirect evidences also point to the basal ganglia and hypothalamus as being the regions of greatest susceptibility (111). On account of relatively specific functional susceptibility of certain cells to appropriate chemical agents, chemical recovery of these agents leads to equivocal results. The Keesers (78, 79) claim to have demonstrated a higher concentration of barbiturates in the hypothalamus than in other parts of the brain. Koppanyi and Dille (92), however, claim that barbital occurs in the same concentration here as in other parts. Thus, the determination of points or regions selectively affected appears to require functional rather than chemical evidences.

Schriever and Perschmann (155, 156) infer that barbiturates act upon the basal ganglia and hypothalamus owing to the effective antag-

[1] The reviewer uses the term "bar'biturate" (pronounced most euphoniously with accent on the first syllable) in the generic sense, even including the acid form.

[2] For a review of the literature prior to 1929, see Lundy and Osterberg (104).

onism by picrotoxin and the relative localization of the latter drug to that region of the cerebrospinal axis.

Respiration. Little can be said as to the direct action of barbiturates on the medulla since such common phenomena as dominant respiratory depression and paralysis in overdosages may well be referable to effects of the drugs on higher centers. Complete but temporary respiratory paralysis can be readily demonstrated to occur in laboratory animals or man with relatively little effect on the vasomotor center, as indicated by negligible changes in blood pressure. Marshall et al. (111) explain the antagonism of barbiturate depression by picrotoxin as a decreased narcosis rather than stimulation of the respiratory center depressed by the hypnotic agent. On the other hand, Marshall and Rosenfeld (110) differentiate depression of the respiratory center from depression of the carotid sinus mechanism. Barbiturates cause a depression of the respiratory center, the activity of which, however, may be maintained by oxygen-want operating upon and through the sino-aortic mechanism. Free oxygen supply, by diminishing oxygen-want, may cause the appearance of greater depression. Thus, barbiturates appear to depress the central respiratory center mechanisms more than the sino-aortic mechanism. Urethane, paraldehyde and alcohol appear to depress, apparently equally, both the sino-aortic mechanism and the respiratory centers.

Burstein and Rovenstine (14) have demonstrated that short and ultra-short-acting barbiturates cause adduction of the vocal cords in cats, a phenomenon frequently seen also in man. This adduction of the vocal cords is abolished by atropine; hence they conclude that the adduction reflex, under parasympathetic innervation, is stimulated by barbiturates, or at least retained in apparently exaggerated form. It may be that this apparent stimulation is but a manifestation of release from control by higher centers or that this particular reflex is especially difficult to suppress.

Circulation. 1. *Vagus.* De Waele (26) reported vagus nerve depression from the effects of the mixture known as "Somnifene." In 1929, Lieb and Mulinos (101) called attention to a depressant or paralytic action on the cardiac vagus mechanism of the rabbit, cat and dog, by amytal. Garry (44) confirmed the observations of Lieb and Mulinos, and added the conclusion that amytal depresses peripheral vagal ganglia. Acetylcholine continues to be active during the period of amytal action; hence he concluded that the post-ganglionic fibers of the vagus are still responsive. Thus, under the conditions of his experi-

mentation, the action of amytal is largely limited to the ganglia. Garry, furthermore, called attention to differences in different barbiturates, in that amytal was effective on the vagus, whereas in ordinary doses phenobarbital was not. These conclusions appear to be confirmed by the work of Koppanyi, Linegar and Dille (93) who demonstrated that both pilocarpine and physostigmine are effective on the cardiac vagus mechanism even though barbiturates have blocked out the effectiveness of electrical stimulation of the vagus trunk. These authors also demonstrated that the effectiveness of different barbiturates differs considerably in that amytal, pentobarbital and pernoston are effective in moderate doses whereas barbital was effective only when dosages were relatively enormous. They did not succeed in demonstrating this depressant effect with phenobarbital in any dosage whatsoever.

Swanson and Shonle (167) failed to demonstrate a vagal depression after pentobarbital, which is likely due to the use of inadequate dosages. It would seem that among the various barbiturates amytal is one of the most effective vagus nerve depressants while phenobarbital is one of the least effective.

Gruber et al. (56) found that thiobarbiturates, if they affect the vagus at all, cause a sensitization of the vagus to electrical stimulation, a phenomenon seen by De Waele, in the early phase of action of "Somnifene." Thus, it may be possible that the sensitization of the vagus by thiobarbiturates is the only manifestation developed by these derivatives owing to the very brief period of their action in contrast to the longer-acting barbiturates which give ample time for the development of depressant effects.

In cold-blooded animals, Gruber et al. (57) have demonstrated that barbiturates, when applied directly to the heart, cause not only paralysis of ganglia, but also nerve endings as well. Possibly differences in local concentrations account for the divergence of views of Gruber and other workers.

2. *Heart.* Roth (150) observed that the hearts of elasmobranchs are stopped by 0.05 per cent solution of pentobarbital, amytal and luminal, whereas barbital failed to cause stoppage. Perfusion of the rat heart by M/500 solutions of each of several barbiturates caused depression (151). Johnston (73) rates the effectiveness of equal concentration of a number of barbiturates in decreasing the amplitude of contraction of the turtle heart, as follows: ortal, amytal, pentobarbital, evipal, phenobarbital, phanodorn, barbital. It will be noted that this order is not strictly parallel to the order of central nervous system toxic-

ity, as indicated by depression or paralysis of the respiratory center (see classification). Johnston (74) demonstrated, furthermore, that barbiturate poisoning of the isolated turtle heart is very difficult to antagonize, the most effective agents tried being glycine and xanthine, which were not good, whereas picrotoxin, metrazol and coramine were poor. Hafkesbring and MacCalmont (59) reported on the electrocardiograms obtained on dogs and cats under the influence of each of the following: nembutal, amytal and barbital. The only cardiac effects of anesthetic doses of each of these agents as shown by the electrocardiogram were an increase in heart rate and a decrease in the common sinus arrhythmia seen in dogs and cats.

Gruber et al. (53, 55) have reported that anesthetic doses of some thiobarbiturates resulted in alternate ventricular rhythm in dogs, rabbits, cats and monkeys. In dogs the P-R interval is shortened. Stimulation of the peripheral vagus may induce an arrhythmia if it had not been present, but if present, vagus stimulation may cause return to normal rhythm. While atropine has no effect on the arrhythmia due to thiobarbiturates, epinephrine may restore the rhythm to normal. Agents that lower blood pressure are also likely to restore the rhythm to normal. Kohn and Lederer (85) studied the EKG in dogs, cats, rabbits and monkeys receiving pentothal and only occasionally did they see abnormal rhythms due to the drug. Ephedrine occasionally restored the rhythm in those few instances in which an abnormal rhythm occurred. In all cases the abnormal rhythm disappeared on termination of anesthesia.

Betlach (5) reported on the EKG in dogs under the influence of amytal and of pentothal and observed no significant alterations. He insists that dogs are so commonly subject to abnormal rhythms that months of observations must be made on each individual animal before drugs are tried in order to know whether or not any irregularities are really due to specific effects of the agents employed. On the clinical side, Volpitto and Marangoni (179) carefully observed seventeen clinical cases of pentothal, thioethamyl and evipal anesthesia and observed no deviation whatsoever from the normal EKG. Cardiac irregularities already present were not altered by these drugs.

It is obvious, therefore, that the question of cardiac irregularities is still open to question and that while they may be seen occasionally in laboratory animals under the influence of thiobarbiturates, the cautions suggested by Betlach and also by Meek and Seevers (115) must be very seriously considered before one is justified in ascribing irregularities

to any one etiological factor, particularly new drugs. To date, therefore, Betlach and also Volpitto and Marangoni appear to have the best of the argument, and until further observations to the contrary have been made, one must conclude that in man, at any rate, no significant alterations in heart action can be ascribed to the specific activity of either barbiturates or their thio-derivatives.

3. *Vasomotor mechanisms.* Bouckaert and Heymans (12) demonstrate a suppression of presso-receptor vasomotor reflexes in connection with the carotid sinus. Nowak (128) claims that amytal and evipal depress the sino-aortic reflexes somewhat less than corresponding doses of pernoston.

In the regulation of vasomotor tone, Nowak and also Bouckaert and Heymans call attention to the varied effects of different barbiturates in depressing the sino-aortic reflex mechanism. Numal and pernoston appear to depress this vasomotor-controlling mechanism more than do amytal and evipal. Thus we have further evidences relative to the different potentialities of the different barbiturates in their modification of activity of peripheral mechanisms. Both the vagus in its peripheral cardiac action and the sino-aortic mechanism in its reflex action are modified differently by different members of the series.

4. *Blood vessels.* Gruber and Baskett (47), and later Gruber and Roberts (48), reported that phenobarbital, administered to laboratory animals under ether anesthesia, caused a drop in blood pressure. Earlier, Tatum and Parsons (171) called attention to the utility of barbital as an anesthetic for dogs since it had the significant property of preserving an approximately normal blood pressure. The inconsistency in reports of these two groups of investigators illustrates how both parties may be right in their observations and yet appear to disagree because of differences in circumstances. According to Bleckwenn (8), barbiturates, acutely administered, generally cause a drop in blood pressure in both laboratory animals and in man. He largely overcame this difficulty clinically by a very slow administration. In the reviewer's laboratory, it has been found that after a lapse of an hour or more the blood pressure becomes stabilized at a level essentially normal. It is not to be forgotten that there are marked individual differences in both man and laboratory animals.

Nomura (127) found the intestinal blood vessels to be dilated, both *in vivo* and *in vitro.* Swanson and Shonle (166) support Bleckwenn to the effect that the fall in blood pressure can be avoided to a very considerable extent by reduction in the rate of injection. Werner (183), in

studying two ultrashort-acting barbiturates, observed that evipal was more prone to produce an acute fall in blood pressure than was pentothal when given to rabbits by intravenous injection in equivalent anesthetic doses. Marx (112) called attention to increased permeability of blood vessels, which may be a factor in causation of cerebral edema so often seen in serious human poisonings by these drugs.

The drop in blood pressure is obviously due to vasodilatation, even demonstrable in perfusion experiments on the brain of the dog. Horsley (70) has observed an abrupt fall in cerebrospinal pressure succeeded by a slow rise. Gruber and Roberts (48) state that barbiturates effect a coronary dilatation when perfused through the cat or rabbit heart.

Gruhzit et al. (58) state that thiobarbiturates are especially prone to produce congestion, stasis, and even hemorrhage in lungs, liver, kidney and the central nervous system. However, clinical experiences with thiobarbiturates indicate that, in a practical way, these manifestations are rarely encountered. This may be due to the fact that man is far easier to anesthetize than laboratory animals, hence requires much smaller doses. Yet the observations of Gruhzit et al. might well be expected to be seen in man in instances of heavy overdosage providing that the patient survives long enough for these manifestations to develop.

Spinal cord. Direct effects on the spinal cord are clearly demonstrated by Dawson and Porter (23) and by Porter and Allamon (133) in their study of the spinal cat. Such preparations receiving evipal, amytal, barbital or luminal reveal a markedly raised threshold of flexion reflexes. Lowering of the threshold by strychnine can be effectively overcome by barbiturates, even to the extent of practical elimination of all crossed reflexes.

Secretory nerves. Stavraky (164) states that amytal, and less readily dial, depress or paralyze the chorda tympani nerve in the dog. Electrical stimulation and also acetylcholine fail to provoke the expected secretion, as after light ether anesthesia, but pilocarpine effects a copious secretion while physostigmine reëstablishes the efficiency of electrical stimulation. These results would lead one to consider amytal to have affected the secretory nerve endings rather than ganglia, adding evidence to Gruber's contention that amytal actually does affect these endings, possibly in addition to ganglionic depression.

Smooth muscle. 1. *Gastrointestinal muscle.* Gruber (49) demonstrated that all of a number of barbiturates studied cause a depression of intestinal, uterine and ureteral muscle *in vitro.* Later, he and asso-

ciates (51) observed a lowering of intestinal tone in Thiry-Vella loops in non-anesthetized dogs to which phenobarbital had been administered. Reynolds (143) observed also a depression of smooth muscle *in vitro* on use of relatively strong solutions of barbiturates, whereas he observed manifestations of stimulation with very dilute solutions. Gruber et al. (52) rather clearly demonstrated that barbiturates in any effective concentration produce depression and that Reynolds' observations of a stimulant action were confirmed only when the alkalinity was allowed to rise. The so-called stimulant dilutions did not produce stimulation if the pH of the solutions was carefully controlled. It is thus obvious that any stimulant action *in vitro* due to alkalinity of strong solutions is overbalanced by the depressant action of the drugs. In dilute solutions the effects are largely, if not entirely, due to alkalinity.

Quigley and Phelps (138) observed that barbiturates produced inhibition of gastric muscle tone equally in normal dogs and in dogs with denervated stomachs; hence the depression is peripheral either on smooth muscle directly or on the peripheral neuromuscular mechanism. This means that the concentrations of the drugs *in vivo* are of such an order of magnitude as to resemble the effects of these drugs *in vitro*. Burstein (15) very recently called attention to a primary depressant action of short-acting barbiturates *in vivo* followed by a prolonged increase in contractions and tonus. Whether this secondary phase is due to a positive stimulant action or is of a secondary nature was not established.

2. *Bladder.* Kolb and Langworthy (88) observed that the barbiturates depress the urinary bladder stretch reflexes of the cat and prevent a sustained vesicle contraction when filling is complete.

3. *Ureters.* Gruber (49, 50) demonstrated a depression of irritability and peristaltic activity of ureters *in vitro*.

4. *Uterus.* Gruber et al. (49, 52) and Boyd (13) observed that barbiturates depressed uterine strips *in vitro*. Berutti (4) found that both pregnant and nonpregnant uteri of rabbits were depressed *in vitro* by relatively strong solutions but unaffected by weaker solutions.

5. *Placental permeability.* Since the placenta is permeated by most crystalloids and many colloidal substances, it is not at all surprising that barbiturates can be demonstrated in the fetus, as reported by Fabre (37) and by Dille (27).

Kidney action. Ogden (130) called attention to the action of amytal in inhibiting water diuresis in dogs during the period of anesthesia. Walton (181) and Gouaux, Cordill and Eaton (46), in studying the 24-

hour urine output, also total blood and urea nitrogen, observed no significant effects of amytal. Any suppression of activity during the short periods of anesthesia was entirely compensated for by a subsequent and transient period of increased action. The kidney function is thus not seriously affected, provided the period of deep depression is not long. If depression is of long duration, partial inhibition of renal action might be expected to be of greater consequence. It is generally recognized that in clinical cases of severe intoxication secretion of urine is apt to be scanty (34).

Blood. In general, the constitution of blood is relatively little affected by the barbiturates when given in moderate dosages. In the rat, which has a much larger relative spleen weight than have other animals, amytal appears to cause a significant acute enlargement of the spleen with an accumulation of erythrocytes, thus withdrawing them from the blood stream, leading to a dilution of the blood. Leucocytes, on the contrary, are apt to increase in number. Splenectomy prevents these changes in blood cell counts (35). Elman, Weiner and Cole (33) observed that the expected dilution of the blood as a consequence of hemorrhage does not occur when the animals are under the influence of amytal. This may be interpreted to mean that amytal causes an accumulation of red cells within the spleen with a dilution of blood. Hemorrhage removes diluted blood. In the course of a short time, red cells return to the circulating blood, thus counteracting the expected dilution due to hemorrhage.

It is generally held that blood sugar is not changed significantly by barbiturates unless depression reaches such a grade as to bring on acidosis. Alkali reserve (CO_2 capacity) and CO_2 content have been said to increase (139), but probably simply due to the alkalinity of the soluble barbiturate. There does not appear to be any significant direct change in CO_2 capacity or CO_2 content other than may be expected from added alkali or by an acidosis from profound depression, involving as it does the respiratory and other centers. Dorfman and Brooks (29) associate their observations of increase in sedimentation rate with dilution of blood due to barbiturates, which condition would indicate some changes in blood viscosity scarcely accounted for by red cell removal.

Metabolism. Anderson et al. (1) have reported their observations on the effects of different barbiturates on basal metabolism. According to them, barbital, ipral, neonal and phanodorn in "usual therapeutic doses" cause some depression of oxygen consumption, whereas amytal and phenobarbital increase it. Shapiro (159), on the other hand,

states that amytal, in intravenous dosages sufficient to produce corneal anesthesia, caused a profound lowering of oxygen consumption. These results are confirmatory of the experiences of Dameshek et al. (22) who ascribed the fall in oxygen consumption to an action on the hypothalamus. Siebert and Thurston (162) observed that amytal, in dosage adequate to "quiet" the animals, produced no significant change in basal metabolic rate. Thus we are forced to believe that the discrepancies between different observers are simply due to differences in dosage and that, in light hypnotic doses, no significant depression below the resting stage occurs, whereas if a more profound depression occurs, a fall in basal metabolic rate is entirely in line with expectations. In the *in vitro* study of barbital on the oxidation-reduction system of methylene blue-ground muscle tissue, Behnecke (3) demonstrated a reduction of oxygen consumption. Other more or less parallel work has been reported by Quastel and Wheatley (137), Jowett and Quastel (76), and Jowett (77). It is yet too early to extend these results to central effects of barbiturates so far as concerns mechanism of action.

Elimination and detoxication. In usual therapeutic dosages, barbiturates are for the most part destroyed in the body of mammals, and only barbital and luminal appear consistently in the urine. Herwick (62, 63) and Shonle et al. (160) demonstrated that pentobarbital, amytal and neonal are not excreted in the urine by dogs receiving amounts causing readily tolerable depression. Koppanyi and his associates (91), however, claim that by the use of the cobalt color reaction all barbiturates can be detected in the urine of animals receiving these drugs. The disagreement between Koppanyi and other investigators appears to reside in either the nonspecificity of the cobalt color reaction or that the dosages were widely different. Both groups of investigators will agree that in dangerously large doses most of the barbiturates will appear in the urine; hence it is likely that there exists a renal threshold for barbiturates. Thus, if the concentration in the blood be low, most of them do not pass through, whereas if the concentration be relatively high, most of them may be demonstrated in the urine. There appears to be some correlation between the stability of the individual barbiturates and their excretability under conditions of moderate dosage. The duration of action may be used as a biological test of stability and, on this basis, barbital, phenobarbital and dial appear to be relatively stable and long acting and appear in the urine in detectable and measurable quantities. On the other hand, those members of the series which on the basis of a relatively short period of action are more unstable, appear

in the urine only under unusual circumstances such as in instances of heavy dosage. A different situation was observed by Pratt (134) who demonstrated that in instances of injury to the liver, e.g., by chloroform or carbon tetrachloride, the short-acting or more unstable barbiturates become longer acting. These conclusions were essentially confirmed by Koppanyi et al. (94) and by Cameron and de Saram (18). Hirschfelder and Haury (65) observed that double nephrectomy had little if any effect on duration of short-acting barbiturates whereas barbital depression was continued until terminus due to the consequences of nephrectomy. Koppanyi et al. (89, 121, 122) reported similarly and added the observation that renal damage through the action of uranium or chromates caused a distinct retardation of excretion of barbital and little change in the course of depression by the destructible barbiturates. These authors also observed that birds and turtles appear to be unable to excrete barbital effectively; hence deep depression due to barbital is permanent whereas recovery from short-acting or destructible barbiturates does occur. Tyson et al. (175) report that concentrated doses of barbiturates (phenobarbital) may be excreted in part also by the mammary glands. If doses be small and repeated, they do not appear consistently in the milk, thus indicating an excretory threshold more or less analogous to renal thresholds.

Kohn and Grimes (86) have made a study of destruction rate of pentobarbital *in vivo* by functional methods which should be similarly applicable to other barbiturates. The method essentially involves determinations of quantities of a barbiturate required to cause death after the lapse of various time intervals after an initial dose. This indirect method is likely to yield data fairly closely paralleling that obtained by complete extraction of carcasses of animals sacrificed at various time intervals after drug administration. Whether or not destruction rates decline in animals in a moribund state or in profound depression has not been clearly established. Koppanyi et al. (95) state that resuscitation of depressed animals by means of stimulant analeptics leads to no significant changes in barbiturate content of the blood and organs of the stimulated animals when compared to the more depressed controls. Thus, the problem of possible changes in rate of destruction according to conditional states has yet to be answered except in instances of parenchymatous damage such as are effected by chloroform or other poisons which particularly affect the liver.

Synergism or summation. Stormont et al. (165) presented convincing evidence that barbiturates significantly increased the anesthetic poten-

tiality of nitrous oxide. With rats as test objects they stated that 85 per cent N_2O-15 per cent O_2 was not anesthetic. When a parallel set of observations was made on rats receiving from 27.5 to 45 per cent of the M.L.D. of barbiturate (dial, neonal, phenobarbital, amytal or barbital), the gas-mixture given to the previous series became effective. Isenberger (71) found that amytal usually considerably reduced the amount of general anesthetic necessary as well as the concentration required for complete anesthesia and relaxation. Kleindorfer and Halsey (82) found that the extinction of the crossed reflex could be accomplished with lower concentrations of ethylene or nitrous oxide when either of these gases was given to animals which had previously received either amytal, dial or allyl-isopropyl barbituric acid. Halsey and Lacey (60) reported experiments on dogs with light ether anesthesia maintained constant by closed system control. When such animals were given either dial or pentobarbital profound anesthesia resulted. If the ether concentration was maintained constant, the animals receiving pentobarbital recovered to a light stage of anesthesia (i.e., ether acting alone) much sooner than those receiving dial. By sharp contrast Calderone (17), in studying the same problem but controlling the blood concentration of ether, came to the conclusion that amytal caused no modification or change whatever in the concentration of ether in the blood requisite to produce any desired stage of anesthesia. Robbins et al. (147), studying cyclopropane, observed that barbital or amytal reduced the concentration of the gas necessary for anesthesia or respiratory arrest. Thus, Calderone appears to stand alone in his contention that pre-anesthetic medication by a number of agents, including barbiturates, does not diminish the amount or concentration of anesthetics required. Unless ether is radically different from cyclopropane the clear-cut report of Robbins et al. is quite convincing that their conclusions and generalizations therefrom are correct and consistent with the practically unanimous opinion of all clinical anesthetists. Possibly some unrecognized analytical error led Calderone to come to his heterodox conclusions. It is too much to expect, with substances as different in action as anesthetics and hypnotics, that addition would be complete. Each substance may well have a characteristic cerebral area of action. Two different substances may have mosaic patterns which overlap to some degree, hence some additive action. Complete overlapping would thus mean identical effects, and identical effects would only be produced by identical substances. Hesse, Baumgart and Dickmann (64) illustrate this point in a quite striking manner when

they demonstrate the potentiation of barbital by phenacetin. Phenacetin is not a hypnotic drug; yet when given with a barbiturate, the effects are clearly accentuated. Phenacetin may be considered to have several "points" of action, one of which is the "sleep center," which is so insignificantly affected as to be unrecognized. Yet when a true hypnotic is simultaneously employed, the points affected in common by the antipyretic and the hypnotic drugs are more affected than would be the case by either acting alone.

Other examples of synergism are reported. Robinson (148) states that calcium potentiates the action of phenobarbital; De Nito (25), that acetylcholine potentiates the action of both magnesium and barbital; Dille and Ahlquist (28), that alcohol increases the action of pentobarbital.

Perhaps the most important practical example of synergism or coaction is the well-known potentiation of barbiturates by morphine. Lundy (105) stated that morphine prolongs the depression produced by amytal. Rowbotham (153) makes the statement that morphine very greatly increases the depressant action of barbiturates. Seevers (158) utilized this principle in using morphine a half-hour or so prior to intravenous administration of pentobarbital for surgical anesthesia in dogs. He found that satisfactory surgical anesthesia could be obtained by the administration of approximately one-half of the dose of pentobarbital that would have been required had morphine not been used. Recovery was quite satisfactory. Vogeler and Kotzoglu (177) used morphine as a predmedication agent prior to use of evipal in rabbits; 100 mgm. per kilogram of morphine one-half hour before caused evipal (20 mgm. per kilogram) to produce a depression lasting sixty minutes, whereas the same dose of evipal without morphine produced a depression lasting for six minutes. Redenz (141) reports confirmation of these results. Pertinent evidence was reported also by Kozelka et al. (98) to the effect that very small doses of morphine given to rabbits very clearly and significantly retarded the destruction of amytal *in vivo*. The cause of this delayed destruction was not established but was assumed to be due to altered circulation of blood through the liver and other abdominal viscera.

Most investigators recognize that when barbital is used as a laboratory anesthetic for dogs (171), a rather long induction period follows even an intravenous injection. In the reviewer's classroom laboratory this inconvenience has been overcome by Seevers and Werner (personal

communication) by the simultaneous administration of pentothal.[3] The animal is anesthetized immediately and continues in this state quite smoothly for hours. Since pentothal alone, in the doses employed, would produce depression for a matter of minutes, a period shorter than the latent period of barbital, it is surprising that the gap is so satisfactorily and evenly bridged.

Antagonism. A study of this phase of the problem has been the subject of a considerable number of contributions, both from the point of view of mechanism as well as practical utility in exigencies as they may arise in clinical practice. Many substances have been used as antagonists to barbiturates in the several manifestations of their actions. In the experimental field, the work of Maloney et al. (108) called attention to the superior efficacy of picrotoxin as a stimulating analeptic. This agent previously had been relegated to the group of outmoded drugs, but in a careful survey these authors observed a superior potency in counteracting the depression effected by barbiturates. Most subsequent investigators have confirmed the observations of Maloney et al. on picrotoxin, especially in more serious grades of hypnotic drug depression. Coramine was recommended by Reese (142) who found that in mild clinical depression it was an effective agent. Caffeine was practically equally effective. Experimentally, coramine as well as caffeine is capable of arousal or partial arousal in animals not profoundly depressed. In the case of deep depression these agents are followed by added depression, and hence are not appropriate to the antagonism in question if recovery is expected. Metrazol has its advocates and comes close to picrotoxin in life-saving capacity and, somewhat like picrotoxin, appears to stimulate much the same centers vitally concerned in profound barbiturate depression. Strychnine, on account of its dominant site of action on the cord and little action on medulla or midbrain, could not be expected to be of much value. Experience has supported this surmise. While strychnine rather effectively antagonizes the depression of the cord produced by barbiturates, when employed to antagonize more vital centers as are concerned with survival, such as the medulla and midbrain, it has little to recommend it. It may be stated that if life is not jeopardized through depression, either coramine, caffeine or strychnine may effect arousal.

The converse situation is rather different. Convulsions produced by

[3] Two cubic centimeters of a solution containing 12.5 per cent barbital soluble and 0.75 per cent pentothal per kilogram body weight.

most convulsants are strikingly controlled by appropriate barbiturates. Strychnine, cocaine, procaine, metrazol, picrotoxin, thujone, insulin in excess, etc., are all effectively controllable within certain ranges of dosages by symptomatic administration of barbiturates.

There are other examples of antagonisms. Meek and Seevers demonstrated that barbital is quite protective against cardiac irregularities caused by ephedrine in dogs. They believe the action of barbital to be due partially to a vagal depression and partially to depression of automatic centers. Hoff and Nahum (66) found amytal effective in preventing cardiac irregularities due to calcium. Acetyl salicylic acid appears to antagonize to a considerable extent the central depression produced by barbital or phenobarbital (Gilman and Barbour) (45). The mechanisms involved in this instance are quite obscure.

By and large, barbiturates appear to be surprisingly effective in controlling convulsions of most organs in that they appear to be selectively depressant on centers or pathways involved in the convulsive processes with, at the same time, less direct action on centers controlling respiration and vasomotor activity. This is illustrated particularly well in the instance of cocaine convulsions. Cocaine certainly poisons medullary as well as other centers, yet the immediate local effect of cocaine on the respiratory and vasomotor mechanisms can be tolerated providing the animals be functionally or anatomically decerebrated. The barbiturates appear to functionally decerebrate without at the same time seriously impairing vital medullary centers (172). The anesthetics, on the other hand, appear equally capable of blocking out convulsive processes, yet they seem to be so much less specific in action that the vital centers are apt to be simultaneously depressed even to the stage of paralysis. Thus, the amount or concentration of ether or chloroform necessary to block out convulsive processes also seriously and dangerously depresses the medulla. In strychnine poisoning the situation is somewhat different in that this drug acts chiefly on the cord with but little evidence of damage to the medulla; hence a general anesthetic would appear to be appropriate in that cord depression or paralysis occurs before a significant depression of medullary centers develops. By the same reasoning, barbiturates, which act chiefly and dominantly on the brain, would not appear to be particularly appropriate even though the cord does partake of the depressive action of the barbiturates as demonstrated and measured by Porter and Allamon.

Classification. Although Vogt (178) spoke of veronal as long in

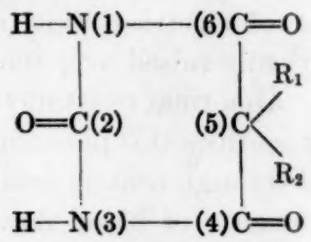
duration and slow in onset of action, phanodorn and amytal with a short period of onset and quick recovery, nostal and pernoston with quickest recovery, Fitch and Tatum (40) were the first to recognize the practicability of using duration of action as a basis of classification. Although there are minor differences in quality of action in different depressant members of the very large series, they are, as a whole, strikingly alike except for duration of effects. These differences in duration are most exactly determined when they are administered intravenously in order to eliminate the factor of differences in rate of absorption, as occurs when they are administered orally. Werner, Pratt and Tatum (184) have emphasized this factor as especially prominent in rabbits. A drug listed as a short-acting agent may behave in these animals as long-acting owing to a large dose, a prolonged period of absorption, and a relatively rapid rate of destruction after absorption. Here the rate of destruction approximates the rate of absorption, thus preventing great accumulation in body tissues. Fulness of the stomach and gastric motility also appear to be important modifying factors. Capacity of the liver and other organs to destroy these drugs must also be considered. Together, these authors proposed four groups into which the depressant barbiturates may fall, viz., long, intermediate, short and ultrashort-acting drugs. Barbital and phenobarbital are representatives of long actors, neonal and dial as intermediate, amytal and nembutal as short, and finally, evipal and pentothal as ultrashort-acting barbiturates. While there are members occupying a borderline status and hence not readily placed, the idea as a whole permits the rational selection of one or other of the ever-increasing series, according to experimental or clinical needs.

Some writers have written of some of the members as having a quick action, i.e., short induction period, whereas others, notably barbital and phenobarbital, as having a long induction period. It so happens that, with the exception of barbital and phenobarbital, practically all of them have a short induction period and hence this basis of classification scarcely suffices to be of significant and practical value.

The classification of duration of action is significantly applicable only to the depressant members of the series. It is well known that there are convulsant barbiturates, as described by Dox and Hjort (30), Shonle and Waldo (161), and Knoefel (84), but these, as yet, have not been found to possess any practically useful properties.

In the experimental field it is extremely difficult to demonstrate

Plan of classification



			(5)		(1)	(2)
			R_1	R_2		
DURATION ↑ increase	LONG	Phenobarbital (Luminal)	USP	ethyl	phenyl		
		Barbital (Medinal) (Veronal)	USP	ethyl	ethyl		
	INTERMEDIATE	Neonal	NNR	ethyl	n-butyl		
		Dial	NNR	allyl	allyl		
		Ipral	NNR	ethyl	isopropyl		
		Alurate	NNR	allyl	isopropyl		
		Nostal	NNR	bromallyl	isopropyl		
		Amytal	NNR	ethyl	isoamyl		
		Sandoptal	NNR	allyl	isobutyl		
	SHORT	Pentobarbital (Nembutal)	NNR	ethyl	l-methyl butyl		
		Phanodorn	NNR	ethyl	cyclohexenyl		
		Ortal	NNR	ethyl	n-hexyl		
decrease ↓	ULTRA SHORT	Evipal		methyl	cyclohexenyl	methyl	
		Pentothal		ethyl	l-methyl butyl		thio

hypnosis or sleep in laboratory animals by use of hypnotic drugs, since the various activities within the laboratory tend to keep such animals awake. Consequently, experimentalists are forced to depend upon symptoms such as incoördination and various stages of anesthesia rather than hypnosis or sleep. Hondelink (69) proposed the use of perching species of birds, finches, canaries, etc., as a means of differentiating hypnotic from anesthetic effects of drugs. This author claims that a bird at the hypnotic level of depression will remain quiet on the perch in a constantly lighted room, whereas if depression reaches the anesthetic levels the bird can no longer remain on the perch but falls to the floor of the cage. These birds may serve as suitable test objects for a comparative measure of true hypnotic potency of drugs.

Richter (146) measured depression effected by hypnotics by means of determinations of electrical skin resistance. He finds that skin resistance is markedly raised over the normal, even over the resistance of normal sleep. Abnormal resistance persists well beyond evident sleepiness. Richter ascribes this phenomenon to sweat gland activity and its central control through centers residing primarily in the hypothalamus. A change in the rate of blood flow is undoubtedly basic to this phenomenon and would appear to be a simpler explanation of the condition described by Richter.

Significance of structure. The relationship of structure to function is now such that certain generalities can be made.

1. It appears established that to be effective both hydrogen atoms on position *5* must be replaced by an alkyl or aryl group.

2. Increasing the length of one or both of the alkyl chains up to 5 or 6 carbons increases the potency, measured by dosage, and diminishes the duration of action.

3. Branched chains are apt to be shorter in action than straight chains.

4. Unsaturated short chains are more active than saturated short chains.

5. Alkyl groups on *1* or *3* lead to a shortening of period of action and tend to produce stimulation more or less in proportion to the length of the chain. Alkyl groups on both *1* and *3* are apt to produce convulsant action (30, 161, 84).

6. Replacement of oxygen on *2* by sulfur increases instability and shortens the period of action (58, 170).

Degradation products produced *in vivo* are as yet unidentified, except in active N-methyl compounds, in which case Butler and Bush (16) report that a considerable amount (40 per cent) of the demethylated analogues are formed.

In the instance of the thio-analogues, pentothal and thioethamyl, it is known that long continued intravenous injection, or repeated intravenous injections, lead to a progressive prolongation of depression (144). This is suggestive of cumulation but such is not the case, according to Kozelka and Hine (99) who have demonstrated that thiobarbiturates exist in the tissues of the dog for only a very short time (e.g., one hour), after which neither the thiobarbiturate nor its oxygen analogue can be found. This is true even at a time, in prolonged administrations, when the animal is still in profound depression, in which case positive identification of the oxygen analogue, if present, could be made without

difficulty. Obviously, then, the long continued use of a thiobarbiturate appears to lead to the accumulation of some unidentified depressant degradation product.

Toxicology. The etiology of poisoning is for the most part self-medication. In many cases, perhaps the greater proportion, the poisoning is intended suicide; others develop a psychic dependence, and hence take the drugs in too large dosages, long after need has ceased, and develop chronic poisoning; finally, there is that peculiar condition spoken of as "automatism" by Richards (145). In this condition the individual, having become accustomed to the use of the drugs, may at some time fail to have deep sleep develop. In this twilight zone, an individual does not exert normal inhibition and reason, and hence mechanically takes all the remaining tablets or capsules. In these cases the coroner's jury pronounces the cause of death as suicide, and so it is, but it should be recognized scientifically as unintentional or accidental suicide. The recognition of this possibility by the medical and pharmaceutical professions might well effect a reduction in the number of accidents of this nature.

Diagnosis of poisoning depends upon history, circumstantial evidence, and finally on chemical analysis of excreta, gastric contents, or tissues and body fluids. There have been developed recently methods of analysis which have given reliable results. The older tests, e.g., Millon's reagent, were non-specific; cobalt color tests are fairly specific qualitatively, if properly used, but rather unreliable for quantitative purposes. Kozelka and Tatum (96) have called attention to the fact that the cobalt color test is not strictly proportional to the amount of barbiturate tested but depends upon at least two variables; hence for unknown amounts of barbiturates, quantification by this method leaves much to be desired in an analytical way. Kozelka et al. (98) have reported a method for quantitative isolation of barbiturates in pure form such as can be determined gravimetrically and identified by melting point determinations. This method is less sensitive for small quantities than the cobalt color test but, in cases of medicolegal character, quantities below the sensitivity range of the gravimetric method are not apt to be of serious or significant toxicological importance. The distribution of barbiturates in tissues has been reported by Koppanyi (95) and by Kozelka (98) and their associates.

The spinal barrier is rather unexpectedly high since barbiturates pass into the spinal fluid very slowly and in very small quantities (41, 97). On the other hand, Purves-Stewart and Willcox (136) maintain that

large quantities are to be found in the spinal fluid of patients poisoned by the use of barbiturates, but they do not reveal the methods of analysis employed. In one of their reports the amount of a barbiturate recovered in an aliquot of spinal fluid examined by their analyst was such that by simple calculation one could prove that most of the barbiturate ingested must have been uniquely collected in the cerebrospinal fluid!

Pathological changes in tissues. Ravn (140) reported a series of studies on the urobilinogen and bile acid tests on some 100 cases in a psychiatric hospital. One-half the cases were given barbital over a period of one to five years. Controls received no barbiturates. Of those receiving barbiturates 64 per cent developed positive tests whereas in the control series but 32 per cent were positive. Thus, the incidence appeared to be higher in the treated series.

Birch (6) reported a case of jaundice due to phenobarbital. Since typical skin lesions appeared, the conclusion was natural that the liver condition was likewise due to susceptibility to phenobarbital. Vaizey (176) reported a case of toxic jaundice due to pentothal. Many writers have reported dermatitis as a common reaction to phenobarbital, while Jontofsohn (75) described in considerable detail, barbituric acid allergy. Maillard and Thomazi (107) reported a case of myalgia and arthralgia as rather clearly due to barbital or luminal. Landor and Salleh (100) saw an instance of widespread paralysis following the use of evipal. Feldweg (38) saw two cases of sudden respiratory paralysis in asthmatics after evipal, which led him to proscribe this hypnotic in asthmatic cases. Mussio-Fournier, Austt and Arribeltz (123) observed a case developing the parkinsonian syndrome and mental disturbances after chronic use of barbital. This observation is affirmed by the Ziskinds (187). These authors state quite positively that phenobarbital is contraindicated in parkinsonism.

Intoxication. Holck and Cannon (68) confirmed the observations of Fitch and Tatum on delayed death of rabbits after nostal and observed in connection therewith the occurrence of parenchymatous damage (fatty changes) particularly in the liver. Kennedy (80) saw parenchymatous degeneration occurring in mice subjected to chronic poisoning by evipal. Seevers and Tatum (157) reported a study of chronic barbital intoxication in dogs covering a period of over three years. Morphological changes were studied for these authors by Dr. Roy Grinker. He described well-marked morphological changes in the brains of these animals, which changes may well be basic to deportmental characteristics. In the dogs there was definite and clear-cut

evidence of a partial tolerance as well as changes in deportment. These deportmental changes and partial tolerance signs were entirely confirmed by Oettel and Krautwald (129). Fitch (39) reported partial tolerance development in rabbits toward a number of different barbiturates. Observations similar to those made by Fitch have been made by a number of investigators (19, 20, 21, 36, 114, 119, 125, 163, 174), whereas only Swanson, Weaver and Chen, in their study of amytal, denied tolerance development (169). Since Fitch, as well as others, found amytal to behave essentially like other destructible barbiturates, the evidence is conclusive that while rate of development and degree of tolerance may vary somewhat in different animals for different barbiturates, they (including amytal) do produce a demonstrable shortening of depression period on repetition of dosage. All of the work reported, except that by Seevers and Tatum and by Oettel and Krautwald, has been too hurriedly completed, i.e., not continued over a sufficiently long period of time to develop real chronic symptoms; hence relatively negative results over a short period are without weight in comparison to positive results obtained after a long period. One could well take the view that negative results would have to endure throughout the entire life of an animal before one could say that chronic poisoning does not occur. Certainly, a few weeks up to half a year would be significant only if positive results were obtained. It is perfectly well known that in the clinical field, chronic poisoning by certain substances may appear even after a period of twenty years.

Habituation. Repeated and continued use of barbiturates induces a condition of habituation or psychic dependence but apparently not a true addiction as exemplified by use of morphine or heroin. (See definitions by reviewer (173).)

Mental disturbances occur during the period of drug-taking and obviously are due to the positive action of the drugs rather than to the cessation of drug-taking or abstinence phenomena. Schmidt (154), on the other hand, describes what he considers to be withdrawal symptoms in his series of eleven human cases who had been taking phenobarbital for six or more years and appeared, therefore, to fit into the category of true addiction. These symptoms were well described by Mussio-Fournier et al. More recently, Weiss (182) and Robinson (149), among others, have discussed this problem. These writers also stress the possibility of irreparable damage to brain and other tissue cells resulting from abuse of these drugs, a condition in man more or less analogous to that described for the dog by Seevers and Tatum.

Treatment of acute poisoning. On the basis of experimental work on the treatment of profound poisoning of laboratory animals, as indicated previously in this review, effective analeptics appear to be limited to two agents, viz., metrazol and picrotoxin. In non-serious poisonings, caffeine, coramine and strychnine appear to be effective in causing arousal but do not raise the lethal dosage of barbiturates significantly. Owing to the uncertainty of dosage a human subject may have taken and his susceptibility thereto, a number of writers have extolled the efficacy of these experimentally weak analeptics. Doubtless these patients would have recovered without the use of these agents if we are to learn from animal experimentation. How much this may be true also for metrazol or picrotoxin cannot be definitely stated, but from such clinical reports as are now available, viz., Bleckwenn and Masten (10), Kohn, Platt and Saltman (87), Kline, Bigg and Whitney (83), Koppanyi and associates (95), and Rovenstine (152), it is clearly apparent that these clinical investigators are convinced that picrotoxin is the most effective and sustaining analeptic so far known for clinical barbiturate poisoning. The argument may be advanced that these cases might also have recovered without the use of powerful analeptics, but owing to uncertainty of exact predictability in any kind of therapy for any disease, a similar logic could be advanced against any therapeutic agent whatsoever. It is safe, therefore, to anticipate that those who are familiar with the evident potency of picrotoxin, properly administered, would elect to use it in serious cases of barbiturate poisoning since a small dosage of a potent drug will work in mild cases, and larger doses may work in serious cases, whereas weak analeptics will likely fail except in instances of minor and non-serious poisoning.

Most writers on this subject stress the importance of simultaneous use of other expedients such as oxygen, gastric lavage, and any other procedures as may be indicated. Cerebral edema requires appropriate attention.

Clinical indications. 1. *Hypnotic.* From what has been presented previously, hypnotic drugs may be of real value in establishing sleep in nervous insomnia. Since these drugs are not analgesic except in overwhelming dosages, pain as a cause of insomnia requires other therapy. The choice of hypnotic must depend upon circumstances (Weiss) such as the possibility that if sleep once is started it will continue more or less normally. For this purpose a short-acting drug would obviously be desirable. On the other hand, if a more or less continuous depression is required in order to effect sleep for a normal period, a longer-acting

drug is required. As a general rule, the short-acting barbiturates are freer from after-effects, i.e., mild depression, than long-acting drugs. If a more or less continuous depression is required through both day and night, a drug such as phenobarbital in repeated small doses very often gives satisfactory results. This is what is accomplished in epilepsy and also in hyperirritability of elderly people. In treatment of pertussis and seasickness, the short-acting barbiturates administered in small doses, symptomatically, appear to be logical and frequently satisfactory. In these instances the drugs are used in subhypnotic dosages.

2. *Pre-anesthetic depressant.* Many surgeons and anesthetists have found that the use of appropriate barbiturates may be satisfactorily employed to quiet a patient prior to anesthesia induction and thereby diminishing "psychic trauma." Many obstetricians are employing these drugs in the first stage of labor where it may not be desirable to too completely obtund labor pains. When used carefully in this way clinicians rarely see any evidence of fetal or infant depression in spite of the fact that experimentally these compounds can be demonstrated in fetal tissues, hence having passed through the placental barrier.

3. *Anesthetic.* The consensus of opinion of most students of anesthesia is that the barbiturates, with two exceptions, do not meet the requirements of anesthetic agents, particularly in regard to analgesia and to possibility of moment by moment control. The two exceptions that may be said to meet one of these requirements fairly closely are evipal and pentothal. These are ultrashort-acting barbiturates and if a "moment" be thought of as a period of five to fifteen minutes, then these agents would qualify in this particular.

4. *Anticonvulsant.* Against convulsions of cerebral origin, barbiturates have been found to be of particularly specific efficiency, as for example against cocaine, thujone, metrazol and picrotoxin, and less specific and satisfactory against cord convulsants such as strychnine. Nonspecific convulsions such as are met with in eclampsia also yield satisfactorily. Since eclampsia is either due to or accompanied by and contributed to by degenerative liver changes, parenchymatous poisons such as chloroform or chloral hydrate, as advocated by Strogonoff, would appear illogical providing there is available an equally effective agent which in itself is not a liver poison. The barbiturates appear to be the best answer available today. American obstetricians are more and more generally employing one or other of the short-acting barbiturates. Even here a certain caution must be raised since in conditions of liver deficiency, destruction of short-acting barbiturates will take place less

readily than in normals, thus leading to a longer action than otherwise would be expected. Since convulsions in themselves are exhausting, it is imperative to keep them from appearing. Other measures are still to be used since convulsions are clearly due to something other than lack of "hypnotics" in the system. In all instances the control of convulsions should be by careful symptomatic administration of an antagonistic drug. The severity of convulsions scarcely suffices to indicate the amount of drug to be given. The practical difficulty of intravenous injection of a hypnotic drug to a person in convulsions may require the use of a volatile anesthetic. This is particularly true of strychnine convulsions.

5. *Neuropsychiatry.* Bleckwenn (7, 9) and Lorenz (102, 103) and their associates first led the way to the psychic analysis of schizophrenias such as dementia praecox. They used first amytal for this purpose but are quite impartial in that they recognize that any short-acting barbiturate will serve the same purpose. Their technique is to administer the barbiturate intravenously and slowly until the corneal reflex is abolished. The patient then is allowed to come out from the effect of the drug. As he comes out there will be a period of a few minutes during which time the physician can converse with the patient. As the effects of the drug diminish further the patient is apt to lapse back into the previously existing noncommunicative state. This would look as though the psychosis were a condition of excessive inhibition, which inhibition could temporarily be removed by a depressant drug. Psychiatrists, no doubt, would express it otherwise, but to a layman in this field the expression *removal of inhibition* conveys the general idea in common language. This view is so expressed by Herman (61). An excellent review of the literature on the use of barbiturates in neuropsychiatric conditions was made by Wagner in 1933 (180).

The use of barbiturates as "truth serum" has appealed to the popular mind although there is nothing either particularly new or certain in its use. It has been known since antiquity that alcoholic intoxication releases the subject from inhibitions and ordinary control, and it appears that any depressant may simulate this action providing the "stages" of anesthesia are not too rapid in their transition. The barbiturates are rather less apt to produce delirium than is scopolamine previously used for this purpose, and hence probably slightly more reliable in action.

The use of these hypnotic drugs for maintenance of prolonged depression in the treatment of psychic disturbances is an old procedure and is still advocated by a number of neuropsychiatrists. Lutz (106), Palmer

and Paine (132), Menzies (116), Jenny (72), and Wilson and Gillman (185) report favorable results, particularly in patients whose abnormalities are of not too long duration. The current practice is to provide the patient with a prolonged and enforced mental rest whereby the habit of psychotic activity may be broken through inactivity.

The use of phenobarbital in treatment of epilepsy appears to depend upon a long continued but mild cortical depression and is still, generally speaking, the drug of choice, though "dilantin," a non-hypnotic drug, has effected a peculiar and unanticipated controlling action (117, 118). It is thus possible that a better understanding of idiopathic epilepsy may be obtained through this interesting, though not entirely safe, hydantoin derivative.

Posology

SPECIES	DRUG	ADMINISTRATION	HYPNOTIC DOSE	ANESTHETIC DOSE	M.L.D.	AUTHORITY
			mgm./kgm.	*mgm./kgm.*	*mgm./kgm.*	
Chimpanzee	Amytal	i.p.		65		Fulton and Keller (43)
	Dial	i.p.		55		Fulton and Keller (43)
	Pentobarbital	i.p.		40		Fulton and Keller (43)
	Pentobarbital (Nembutal)	rectal		25–30		Elder (32)
Rhesus	Dial	i.p.		40		Fulton, Liddell and Rioch (42)
Horse	Pentobarbital	i.v. or i.p.		9.5		Abbott Laboratories
Cattle	Pentobarbital	i.v. or i.p.		9.5		Abbott Laboratories
Sheep	Pentobarbital	i.v. or i.p.		28		Abbott Laboratories
Swine	Pentobarbital	i.v. or i.p.		28		Abbott Laboratories
Dogs	Amytal	i.v.		58		Holck and Kanan (67)
	Amytal	i.p.		50–70		Mulinos (120)
	Amytal	i.p.		34–44		Hafkesbring and MacCalmont (59)
	Amytal	i.v.		45–60		Page and Coryllos (131)
	Amytal	i.p.		50		Rakieten et al. (139)
	Barbital	oral		250		Tatum and Parsons (171)
	Barbital	i.v.		250		Unpublished data (Tatum)
	Barbital	i.p.		180		Hafkesbring and MacCalmont (59)
	Dial	i.p.		50		Rakieten et al. (139)
	Dial	i.p.		50		Fulton, Liddell and Rioch (42)
	Nostal	oral		20–35		Boedecker and Ludwig (11)
	Ortal	i.v.		40		Gruber and Brundage (54)
	Pentobarbital	i.v. or i.p.		28		Abbott Laboratories
	Pentobarbital	i.p.		45–55		Mulinos (120)
	Pentobarbital	i.p.		22–25		Hafkesbring and MacCalmont (59)
	Pentobarbital	i.p.		30		Rakieten et al. (139)
	Pentobarbital	i.p.		22–45		Bazett and Erb (2)
	Pentothal	i.v.		15–20		Pratt et al. (135)
	Pernoston	i.p.		40–60		Mulinos (120)
	Phenobarbital	i.p.		20		Rakieten et al. (139)
	Seconal	i.v.		25		Swanson (168)
Cats	Amytal	oral			100	Eddy (31)
	Amytal	i.p.		60–80		Mulinos (120)
	Amytal	i.p.		24–25		Hafkesbring and MacCalmont (59)
	Barbital	oral			280	Eddy (31)
	Dial	i.p.		50		Fulton, Liddell and Rioch (42)
	Ipral	oral			140	Eddy (31)
	Neonal	oral			84	Eddy (31)
	Pentobarbital	i.p.		18		Hafkesbring and MacCalmont (59)
	Pentobarbital	i.p.		22–45		Bazett and Erb (2)
	Pentobarbital	i.v. or i.p.		28		Abbott Laboratories
	Pentobarbital	i.p.		45–55		Mulinos (120)
	Phanodorn	oral			120	Eddy (31)

Posology—Concluded

SPECIES	DRUG	ADMINISTRATION	HYPNOTIC DOSE	ANESTHETIC DOSE	M.L.D.	AUTHORITY
			mgm./kgm.	*mgm./kgm.*	*mgm./kgm.*	
Rabbits	Amytal	i.v.		45	75	Holck and Kanan (67)
	Amytal	i.p.		55–60	90	Fitch and Tatum (40)
	Barbital	i.p.			225	Fitch and Tatum (40)
	Evipal	i.p.		70		Maloney and Hertz (109)
	Ipral	i.p.			110	Fitch and Tatum (40)
	Narconumal	i.v.		20	50	Demole (24)
	Neonal	i.p.			115	Fitch and Tatum (40)
	Nostal	i.p.			120	Fitch and Tatum (40)
	Pentobarbital	i.p.		30–40		Mulinos (120)
	Pentobarbital	i.p.		40	65	Fitch and Tatum (40)
	Pentothal	i.v.		20	35	Pratt et al. (135)
	Pentothal	oral		360	600	Pratt et al. (135)
	Pernoston	i.p.		90–100		Mulinos (120)
	Pernoston	subcut.		50	160	McCarthy (124)
	Pernoston	i.p.			75	Fitch and Tatum (40)
	Phanodorn	i.p.			130	Fitch and Tatum (40)
	Phenobarbital	i.p.			150	Fitch and Tatum (40)
Guinea pigs	Evipal	i.p.		100		Kennedy (80)
	Pentobarbital	i.p.			45–60	Carmichael and Posey (20)
Rats	Amytal	subcut.		100–250		Nicholas and Barron (125)
	Amytal	i.p.			115	Fitch and Tatum (40)
	Barbital	i.p.			300	Fitch and Tatum (40)
	Evipal	i.p.		100		Maloney and Hertz (109)
	Neonal	i.p.			135	Fitch and Tatum (40) Nielsen et al. (126)
	Ortal	i.p.		100–110		Gruber and Brundage (54)
	Pentobarbital	i.p.			75	Fitch and Tatum (40)
	Pentothal	i.p.		80		Pratt et al. (135)
	Phenobarbital	i.p.			155	Fitch and Tatum (40)
	Seconal	i.p.		40		Swanson (168)
Mice	Evipal	i.p.		95	190	Kennedy (80)
Fowls	Barbital	i.v.	150		225	Koppanyi et al. (90)
	Dial	i.v.	60			Koppanyi et al. (90)
	Neonal	i.v.	60			Koppanyi et al. (90)
	Pentobarbital	i.v.	25			Koppanyi et al. (90)
	Pernoston	i.v.	20			Koppanyi et al. (90)
	Phenobarbital	i.v.	100			Koppanyi et al. (90)
Chicks	Pentothal	i.v.		20		Klein, B. E. (personal communication)
Pigeons	Barbital	i.m.		30		Winiwarter (186)
	Evipal	i.m.		4		Winiwarter (186)
	Pernoston	i.p.		30–40		Mulinos (120)
	Phenobarbital	i.m.		12		Winiwarter (186)
Finches	Barbital	i.m.		125		Hondelink (69)
Turtles	Barbital	i.v.		200–300		Koppanyi et al. (90)
Frogs	Evipal	i.p.			30–80	Kennedy (80)
	Pernoston	subcut.		60	150	McCarthy (124)
Fish	Amytal	i.p.		40–54	60	Keys and Wells (81)

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MUSCLE HEMOGLOBIN

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About one-fourth of the body's hemoglobin is not able to circulate in the blood, being locked up in the muscle fibres. This large fraction of the body's pigment has received but scant attention at the hands of physiologists and biochemists until quite recently, and even today it is the subject of less than one hemoglobin paper in twenty. Yet muscle hemoglobin is an exceptionally interesting substance in the beautiful simplicity of its physico-chemical behavior, in its physiological rôle, and in the insight which it is able to give us into aerobic muscular activity. The reason for our apathy is not far to seek: the pigment could neither be studied conveniently *in situ* nor easily extracted in a purified form. At the same time blood hemoglobin's obvious function as an oxygen carrier, coupled with the tempting ease with which it can be obtained, made it an all too successful competitor as an object of research. Since the isolation and crystallization of muscle hemoglobin by Theorell in 1932 (40), our knowledge has rapidly increased, though there is still a host of problems concerning the formation, disappearance, and certain aspects of its behavior in the body which have not yet even been tackled.

Nomenclature. It is fortunate that so little confusion of meaning has arisen from the multiplicity of names and symbols which have been attached to this substance; as long as this remains the case the matter of nomenclature may be regarded as of minor importance. *Muscle hemoglobin* or *myohemoglobin* are satisfactory terms in that they imply nothing more than the "hemoglobin occurring in muscle," but both words are somewhat long and cumbersome to use. *Myoglobin*, proposed by Günther in 1921, when he became convinced that the muscle and blood pigments were different, has the advantage of being more compact. The present author slightly prefers the longer term because of his desire to retain one word which will embrace that large family of substances, no matter where they are found or what their molecular weights, which consist of conjugated proteins 1, capable of combining reversibly with oxygen and carbon monoxide; 2, capable of being

oxidized to a "met" compound; 3, possessing a characteristic type of spectrum, and 4, whose prosthetic group is reduced heme (from protoporphyrin). This family includes the blood pigments of all vertebrates, the invertebrate erythrocruorins of Svedberg, and the muscle pigment. It excludes chlorocruorin, which has a different heme group, the cytochromes and helicorubin, which do not combine reversibly with oxygen, hemerythrin, which does not contain heme, and the copper-containing hemocyanins.

Myochrome of Mörner is now never used. MacMunn's *myohaematin* is now known as cytochrome. MacMunn also saw muscle hemoglobin, but did not distinguish it from the blood pigment.

Notation. In this paper MHb = muscle hemoglobin; Hb = blood hemoglobin.

1. Occurrence. Muscle hemoglobin is generally found in large quantities in those muscles requiring slow, repetitive activity of considerable force. The highest concentrations are found in muscles whose complete cycle of activity takes the order of a second, and whose action must be maintained over long periods. Examples are the heart muscles of larger mammals, breast muscles of the larger flying birds such as the pigeon, and leg muscles of running animals such as the horse and the dog. Muscles which regularly contract more than two or three times a second usually have much cytochrome and but little or none of the hemoglobin. Examples of these are the wing muscles of flying insects (flies, wasps, bees, etc., which may contract with a frequency as high as 300 per second), and hearts of small birds and mammals such as canaries and mice. On the other hand, muscles which are called upon for repetitive activity of no very great power, such as the heart muscles of frogs, or for intense contractions with large intervals of rest between, such as the leg muscles of frogs, contain considerable quantities of cytochrome but no muscle hemoglobin.

This principle of distribution is not, however, quite universal. The occurrence of MHb among invertebrates is puzzling and sporadic. It is true as Ray Lankester pointed out (23) that some of the most active invertebrate muscles, such as the pharynx of the gasteropods *Limnaeus* and *Paludina*, do contain hemoglobin, even though the blood of these animals is blue or colourless; on the other hand, the great majority of invertebrate muscles, including most invertebrate hearts, contain no trace of this pigment though the more active ones are rich in cytochrome. All active muscles contain considerable amounts of cytochrome, whether they contain the muscle hemoglobin or not.

However, in a red muscle MHb may have a concentration 50 times that of the cytochrome, and the latter is thus easily masked. (For the relative occurrence of these two pigments see Keilin, 18.) A curious distribution of the pigment occurs in newborn infants, which contain considerable amounts of it in their diaphragms but none in their hearts, although the heart muscle is earlier put to work (Jonxis, 17).

Among mammals and birds we find a close correspondence between MHb concentration and the tissue's requirements for vigorous but slow repetitive activity; this principle may be followed not only from muscle to muscle within one species, but also from species to species, as well as from time to time in the life of a single individual. The physiological and chemical differences between red and white muscle have been thoroughly reviewed by D. M. Needham (28). Where both kinds occur in the same muscle, as for example the soleus (red) and gastrocnemius (pale) of the cat, it is found that the twitch of the red muscle is more prolonged, the tetanus is much more marked, its maximum is achieved at a much lower frequency of stimulus, and it can be maintained for a much longer time. To this point we will return when we consider the physiological rôle of the pigment.

Effect of age and activity. The same muscles may vary widely in redness from individual to individual and from time to time, depending upon the amount of use to which they are being put. Much the most complete and careful study of these variations was made by Whipple and his collaborators (47, 48, 49) on dogs.[1] Some of the results are given in table 1, from which it may be seen that whereas newborn pups have very pale muscles, they develop redness with increasing age and activity. If the motor nerve to a red muscle is cut, it is found that there is rapid depletion of the MHb (5, 48), although bleeding has but a slow and small effect. "The demands of the active muscle for its

[1] Estimates of the concentration of MHb made before about 1925 must be accepted with caution, because many of the earlier workers were unaware of the fact that much of the redness of muscle may be due to cytochrome; up to 1932 many writers regarded the muscle and blood pigments as being identical and not to be distinguished spectroscopically. Whipple was among these, but his work is reliable because he freed the muscle from Hb by simultaneous perfusion and bleeding, and because his method of extraction left cytochrome in the muscle mince. Watson (46) believes that Whipple's values were too high because of imbibition of fluid during extraction. If this objection is valid, it will not affect the significance of the *relative* results which are the important ones for our present purpose.

hemoglobin evidently are honoured as meticulously in the internal metabolism clearing house as are the demands for the blood-forming organs"—Whipple.

Abnormal appearance of MHb in urine. In certain pathological states, MHb appears in the urine. This condition has been studied very fully in the horse, where it is associated with extreme muscular stiffness (lumbago, myohemoglobinuria, Kreuzlähme) (Carlstrom, 7). The author knows of one human case in which the symptoms were

TABLE 1

MHb concentration in dogs in milligrams per 100 grams tissue

a. Age variations

AGE	MHb		Hb BLOOD
	Legs, back	Heart	
2 months	107	125	8,300
4 months	205	222	9,400
Adult	815	480	18,200
Old	680	480	15,700

b. Variations with exercise

	MHb		
	Diaphragm	Heart	Legs, back
Small quiet poodle	387	296	391
Average healthy dog	723	450	700
Hunting dog		770	1051

Data from Whipple (1926). All values are given in milligrams of pigment per 100 grams tissue or blood. Each figure of table 1a is the average for several dogs. The values will be of the same order for horses and cattle. For comparison, the concentration of cytochrome c in horse's heart (a rich source) is c. 40 mgm. per 100 grams (Keilin and Hartree, 20).

nearly identical with those of the equine disease; it was completely different from paroxysmal hemoglobinuria, though large quantities of pigment (MHb, not Hb) were excreted. In this case the patient's plasma potassium went up during exercise by about double the normal rise, suggesting that his muscle membranes were made unusually permeable during activity.

2. PROPERTIES. A comparative list of the principal known properties of MHb and Hb are given in table 2; a critical estimate of their probable reliability, the methods by which they were discovered, and

TABLE 2

Comparative properties of muscle hemoglobin and blood hemoglobin. For qualifications, see text

	MHb	Hb	REFERENCES
Some common properties			
1. Prosthetic group	Ferroheme		37, 32
2. Iron content	0.345%		40
3. Reversible combination with	O_2, CO		14, 43, 44
4. Reversible redox system Fe″, Fe‴			
Spectroscopic			
5. Position, alpha band, oxy-	581 mμ	577 mμ	27, 29, 21, 18, 31, 42
6. CO-O_2 span	3.1 mμ	4–6 mμ	29, 33, 42
Gas-binding			
7. Shape of dissociation curve	Hyperbolic	Sigmoid	14, 44, 15
8. Oxygen affinity	Very high	Moderate	
pO_2, ½ sat'n. pH 7.4, 37° horse	3 mm.	22 mm.	44
9. pH effect on diss'n curve	Small	Moderate or large	44, 15
10. Relative CO/O_2 affinity	20	500	33, 43
11. Kinetics, pH 7.4 20°C. horse:			
O_2 combination, millimol^{-1} sec^{-1}	19,000	4,000	24
O_2 dissociation, sec.$^{-1}$	37	40	
CO combination, millimol^{-1} sec^{-1}	300	130	
CO dissociation, sec.$^{-1}$	0.04	0.004	
Miscellaneous distinguishing properties			
12. Molecular weight	17,500	68,000	39, 41
13. Solubility, horse	Very great		26
14. Rate, alkali denaturation	Very slow		12
15. Precipitin reaction	Specific	Specific	13
16. Ease of oxidation to "met"	Very easy	Less easy	40
17. Iso-electric point, horse	6.99	6.78	40

some supplementary material are given in the section which follows. In general it may be said that all properties which have been found to be identical in *all* blood hemoglobins are also shared by the

muscle pigment. On the other hand, where the blood hemoglobins have small quantitative differences among themselves, the muscle hemoglobins have a range of values so distinct from that of their blood analogues as to justify the creation of a sub-class. This is most marked in properties (5, 6, 7, 8, 9), where half a dozen species have been studied comparatively.

The preparation of the pure crystalline material by Theorell was a great step forward, as it enabled us to be sure that the distinctive properties which had been observed in solutions containing MHb were not due to impurities or to the admixture of some of the blood pigment. Full details of his method of preparation, which has been repeated successfully by several other workers, are given in Theorell's paper (40). The essential steps are the water extraction of the pigment from minced muscle tissue (perfused horse's heart gives the best results), the precipitation of foreign proteins by basic lead acetate, removal of traces of Hb by dialysis against saturated ammonium sulfate solutions, and the final crystallization by further dialysis. Practically all of the properties listed in the table can be observed in non-purified extracts as well as in the crystallized preparation, but until this identity was proved, all our knowledge of the pigment was uncertain.

Table 2: authorities and supplementary material. (1, 2, 3 and 4) now command universal agreement. There is no need for further discussion.

(5, 6). A complete spectrophotometric study of crystalline horse muscle hemoglobin, including all its common derivatives, oxy, CO, reduced, "met" has been made by Theorell (42). It includes the spectral range from 250 mu to 650 mu.

The position of all the visible bands are displaced to the red, not merely the alpha band. This shift is due to the globin part of the molecule, as the hemes of MHb and Hb are identical. Furthermore, the recombination of heme from either source with globins, always gives the band position corresponding to the original source of the globin (33).

The 40 Ångstrom displacement to the red is a property of the pigment itself, and is not an artefact of extraction, since it is found *in situ* in the living tissue (29). Though there are some differences as to the exact location of the position of the alpha band maximum from species to species, and from one observer to another, all authors place it between 5800 and 5825Å (42, 31, 38, 29, 46, 21, 25), whereas the corresponding band in vertebrate Hb is never further to the red than 5780 Å.

The only invertebrate whose blood and muscle pigments have been comparatively studied is *Ascaris*, for which Keilin found the MHb alpha band position at 5802 A., and for the Hb band 5785 Å. The shift here is in the right direction but is smaller than in mammals.

The difference in band position gives us the most convenient methods of identifying and estimating MHb in the presence of Hb, as it is found that a very simple relation exists between this quantity, as determined with a Hartridge spectroscope, and the relative amounts of the two pigments in a mixture (Watson, 46, 25). A spectrophotometric procedure has also been given, using the relative absorption coefficients at 577 mu and at 580 mu as the criterion (38).

When oxygen is replaced by CO the bands shift by a much smaller amount for MHb than for Hb. The range for various Hb's is from 46 to 62 Å (1), that for MHb from 29 to 36 Å, depending on the species (33).

A slightly lower value for the absolute absorption coefficient has been found on several occasions for MHb than for Hb. These differences are probably not significant, being little greater than the errors of the experimental methods by which they were obtained.

(7, 8, 9) Several gas-binding properties were worked out independently and at nearly the same time by R. Hill (14, 15) in Cambridge, and H. Theorell (40–44) in Stockholm, using different preparations and different methods. Hill used dilute water extracts (0.05 per cent–0.1 per cent) from perfused hearts of dog, sheep, ox and goat; the gas equilibria were determined entirely in a Thunberg tube, using a simple and elegant method, the degree of saturation being determined spectroscopically. Theorell used his crystalline horse heart preparation, and measured the dissociation curves of concentrated solutions (2.5 per cent to 6.0 per cent) with the Van Slyke apparatus, using a technique in which the entire equilibrium process as well as the measurement of combined gas was carried out in the bulb of the machine. Hill always reached his equilibrium point from below, Theorell from above. In spite of all these differences in technique, the two workers were in essential agreement, finding always hyperbolic curves, high oxygen affinity, and small effect of pH on affinity, which is by itself strong presumption of the correctness of their findings. Theorell also investigated the effect of temperature on the dissociation curve, and found a Q_{10} of 2.58, corresponding to a heat of reaction of 17,500 calories, which is considerably less than the corresponding values for blood hemoglobin. (10) CO/O_2 affinity. Theorell at pH 6.95 obtained the following values: 20°C: 19.3; 37°C: 13.8; 15°C:(extrapolated) 28.1.

(11) Kinetic results were obtained by the streaming flow method of Hartridge and Roughton, using crystallized MHb from horse's heart. The "O_2 combination" reaction is the fastest one involving biological substances which has been directly measured.

(12) Theorell's original molecular weight determinations (41) gave a value fo 34,000 or mixtures of 34,000 and 68,000, but recent results obtained by the same ultracentrifugal method and in the same laboratory (39) give values of 17,200 and 17,500. All agree in finding average molecular weights less for MHb than for Hb, which explains the rapidity with which MHb when injected into the blood stream is passed through the kidneys into the urine. This had been first reported in 1902 by Camus and Pagniez (4) for rabbits and dogs; it has since been extended to horses (6, 7), and confirmed on the dog (13), whose threshold of renal excretion for MHb is about $\frac{1}{5}$ that for Hb.

(13) The solubility of COMHb at pH 6.6 and 25°C. was measured by Morgan (26), who found that $\log S = 8.00 - 0.94\left(\frac{\Gamma}{2}\right)$, where S = solubility in grams per litre, and $\frac{\Gamma}{2}$ is the ionic strength per litre. COMHb is quite soluble at pH 6.6 in buffer solutions up to 3M phosphate, where COHb has a solubility less than 1 part in 1,000,000. The great difference in solubility provides a method for quantitative separation of MHb from Hb.

(14) Haurowitz, using material from humans, found MHb very much more resistant to alkali denaturation than Hb. This difference has been confirmed by Brinkmann and Jonxis, who now use the principle as a method of isolating and purifying MHb.

(15) The precipitin reaction of MHb and Hb from the same animals is specific.

(16) All observers agree as to the much greater ease of oxidation of MHb. The author knows, however, of no published results on the redox potential of the system MHb-met MHb.

(17) There is not good agreement on absolute values, but several workers have reported that the iso-electric point of MHb is 0.1 to 0.2 pH unit more alkaline than that of Hb.

3. PHYSICAL CHEMISTRY. *MHb as a "primitive" respiratory pigment.* Muscle hemoglobin is our best example of a respiratory pigment whose behavior is simple. It possesses the hyperbolic dissociation curve about which blood physiologists have been dreaming for 40 years but have found, alas, so seldom in the laboratory. It contains only one heme

group per molecule, the molecular weight being 17,200. It has a nearly negligible hydrogen ion effect. Anthropomorphically speaking, muscle hemoglobin appears to be the first attempt of a creator who was primarily a physical chemist to produce a respiratory pigment, before he became aware of the biological desirability of putting a crimp in the dissociation curve, or of giving the pigment a Bohr effect.[2] It is an ideal substance on which to test those minimum mass action assumptions, which are the basis of all oxygen-binding theories, and on which the intermediate compound hypotheses of Adair and Pauling rest.

The simplest specification which we can imagine for a respiratory pigment is that its combination with oxygen should take place according to the equations:

$$MHb + O_2 \rightleftarrows MHbO_2$$

$$\frac{[MHbO_2]}{[MHb]\,[O_2]} = K$$

in which the combination process is a bimolecular one, while the splitting-up is unimolecular. For such a substance the equilibrium dissociation curve can only be a rectangular hyperbola passing through the origin and asymptotic to the horizontal line of 100 per cent saturation. There can be any number of such curves, but they are all of the same shape and differ from each other only in the amount by which they are stretched out along the horizontal or oxygen tension axis. Each curve is defined by a single, unequivocal equilibrium constant, K, the expression for the curve being:

$$\text{degree of saturation} = \frac{Kp}{1 + Kp}$$

where p is the oxygen tension. Curves of this shape have been found to occur with MHb, no matter from what animals they have been obtained. They do not occur with Hb, except as a result of rather rough handling (see Forbes and Roughton, 8, and Roughton, 35, p. 456, for critical surveys), but they have been reproducibly found in the dialyzed hemocyanins of *Helix pomatia*, *Limulus*, and *Busycon* (30). These hemocyanins share with muscle hemoglobin the interesting property

[2] The sporadic occurrence of MHb among lower animals and its absence from many of those possessing blood hemoglobins of a mammalian type militate against any serious theory for the evolution of the hemoglobins along these lines. There is no evidence that MHb appears earlier in evolutionary development than Hb.

of having their dissociation curves but little affected by changes in pH. This presumably means that in all these pigments the point at which the oxygen is attached to the pigment molecule is sufficiently removed from the acidic and basic ionization groups to be little affected by them. That the two properties (hyperbolic curve and small Bohr effect) can be independent, however, is shown by the example of the worm *Urechis caupo*, whose blood hemoglobin possesses the sigmoid curve but no effect of pH.

Kinetic experiments. A very rigorous test of the mass action interpretation of the hyperbolic curve consists in measuring the rates at which combination and dissociation processes take place, and comparing the quotient of the two opposing velocity constants with the equilibrium constant. Agreement was in fact found within the limits of experimental error both for O_2 and CO (24). The principal onus for the high oxygen affinity of MHb was found to rest with the "on" reaction, which is over four times as fast as the corresponding one for Hb, half completion of the reaction being reached in 0.0004 sec. under the conditions of the experiment. The small effect of pH on the equilibrium dissociation curve was also reflected in the kinetic experiments, but here it was the "splitting-off" reaction which showed the difference: Hb alters its dissociation rate tenfold in a pH range of 2.5 units, MHb showed no change in rate under the same conditions.

Significance of the molecular weight. A hyperbolic curve, and *only* a hyperbolic curve, is to be predicted for a "baby" respiratory pigment, i.e., one which has only room for one molecule of oxygen per molecule of pigment. A curve of any other shape would seal the fate of all attempts to apply mass action considerations to hemoglobin right at the start. But MHb is the only baby pigment known, and the confirmation of the expected form of its curve is significant experimental evidence that the more complicated mass action theories (Hill, Adair, Pauling, etc.) are proceeding on correct basic assumptions.

The converse proposition does not hold good, for a hyperbolic curve does not necessitate a baby pigment. Such a curve may occur with a molecule containing any number of oxygen-binding groups provided simply that these different groups are independent of each other. This is the condition occurring in the giant hemocyanin molecules, which possess hyperbolic curves but have molecular weights of several millions.

Relation between CO/O_2 affinity and spectral "span." The small quantitative differences which characterize the various hemoglobins have defied all attempts to bring them into one orderly system, with the

exception of the one remarkable relation discovered by Anson, Barcroft, Mirsky and Oinuma (1) between gas-binding and spectroscopic properties. These authors found that the shift in position of the alpha band when CO replaced O_2 was larger for those hemoglobins which had a greater affinity for CO relative to O_2. For example, a dog whose partition constant, M,[3] was 550 had a "span"[4] of 61 Ångstroms, while a rabbit with a partition constant of 150 had a span of 48 Å. Furthermore, the data for all thirteen species examined showed that log M was directly proportional to the span. As one of the authors pointed out (2), however, the smallest span measured was 46 Å and the largest 62 Å, while the experimental error was large enough to make the straightness of the line somewhat questionable. Theorell's very careful data on MHb (43), and Roche's previous observations (33) enable us to test the phenomenon over a larger range, the span here being 32 Å and the partition constant 28.1. This point lies exactly on the original straight line, now extrapolated to over twice its previous length, and, in the view of the present writer, this confirmation establishes the relationship as a fundamental property of hemoglobin. It was pointed out in the original paper that the band displacement is proportional to the change in free energy of the system (since log K is a measure of this quantity), but unfortunately no more detailed explanation of this most interesting phenomenon in terms of physical chemistry has yet been made.

4. THE RÔLE OF MUSCLE HEMOGLOBIN IN THE BODY. Muscle hemoglobin might serve the body in three capacities:[5] 1, as an agent in oxygen transport; 2, as an intracellular catalyst; 3, as an oxygen store.

1. Oxygen transport. Muscle hemoglobin could conceivably circulate inside the cell in the same manner as the blood circulates in the body, picking up oxygen at the periphery and giving it up to enzymes

[3] "Partition constant" = relative concentrations of O_2 and CO in equilibrium with Hb, which is 50 per cent in the form O_2Hb and 50 per cent in the form COHb =

$$M = \frac{[O_2]\,[COHb]}{[CO]\,[O_2Hb]}.$$

[4] "Span" = difference in position of the maximum of the alpha band between COHb and O_2Hb as measured e.g. with the Hartridge reversion spectroscope.

[5] The present treatment owes much to chapter V of Barcroft's *Architecture of Physiological Function* (1934). The considerable number of new experimental facts which have come to light in the last five years provide the only excuse for this supplement to that stimulating and readable chapter.

in the cell interior. Two circumstances make this rôle extremely unlikely: the lack of any apparent mechanism for circulation inside the muscle fibre, and the very high rate of diffusion over small distances, which would render such circulation of small value. Second, it might remain stationary itself but be of aid to the flow of oxygen molecules, by combining with them and releasing them in an oriented or activated state, so that they could be more readily handled by the cellular oxidases. But in this case MHb is by definition a catalyst. If, on the other hand, it combines with oxygen in a thermodynamically reversible manner, the released molecules of oxygen must be of precisely the same reactivity as those which have not yet been bound; if there were now a uniform flow of oxygen to the interior of the cell, the presence of the pigment in such an equilibrium state would be of no aid, for its removal would bring no change in the number of oxygen molecules made available each second for the use of the cell. True, in periods of fluctuating supply or demand, the pigment would help to even out this uneven flow, but in this case it would be acting simply as an oxygen store. The problem of oxygen transport has thus been transformed either into one of catalysis or into one of storage—transport in time instead of space—and to these two possibilities we will now turn.

2. Catalysis. There is no direct evidence as to whether muscle hemoglobin acts catalytically in the muscle or not. No specific enzymatic activity has been ascribed to it: none have been investigated. It is unfortunate that we do not even know whether it is a more effective accelerator of the oxidation of fats than blood Hb, or how the catalase activites of the two pigments compare.

In one group of properties MHb occupies a strikingly intermediate position between Hb and oxidase systems. There is an often-remarked analogy between the reversible binding of oxygen by blood pigments and of a substrate by its enzyme according to the Michaelis theory. The geometrical configurations are similar, and a number of physicochemical properties may be quantitatively compared. Four of these are listed in table 3 (to which the corresponding figures for catalase have also been added). It is seen that MHb is more like the oxidase than Hb in three out of four of these properties: its oxygen affinity, its speed of combination, and its relative CO/O_2 affinity. Finally, MHb is much more easily oxidized to the met form than Hb; since hemin catalysts probably owe their activity to their easy alternation between the bivalent and trivalent states, the muscle pigment in this property too is more like the enzyme than is the blood pigment. It appears to

be a sort of half-way station or connecting link between oxygen carriers and oxygen catalysts.

It is just conceivable that MHb might suddenly reduce its oxygen affinity at a moment of oxygen need, perhaps by the alteration of surface forces associated with contraction, in this way providing an extra supply just when it is wanted. This would be catalysis of a rather special sort. It is, however, highly improbable and there is some evidence against it.

TABLE 3*

Comparison of some properties of blood hemoglobin, muscle hemoglobin, oxidase, and catalase

Neutral pH, room temperature, figures only approximate

PROPERTY	Hb HORSE	MHb HORSE	OXIDASE YEAST, COCCUS, ETC.	CATALASE, HORSE LIVER
Substrate concentration at ½ saturation, millimols	1.7×10^{-2}	1.5×10^{-3}	5×10^{-5}	2.5×10^{-1}
Combination velocity constant, 1/sec. millimol	7×10^{3}	1.7×10^{4}	$>2.3 \times 10^{6}$	$>7.6 \times 10^{3}$
Dissociation velocity constant, 1/sec.	c. 40	c. 40	>c. 5	3×10^{5}
O_2/CO at equipartition (M)	500	18	1/10	
Oxidation state of Fe	Bivalent, oxidizable	Bivalent, easily oxidizable	Bivalent and trivalent alternating ?	Bivalent and trivalent alternating ?

* The data for this table were obtained from the following sources: Hb affinity (9); MHb affinity (44); oxidase affinity (45); catalase affinity (11); velocity constants: Hb, MHb (24); Atfe (45); catalase (50); M (2, 43).

3. *Store.* The store theory is supported both by the qualifications of the pigment and by direct evidence. An effective store must have *a*, sufficient capacity; *b*, be able to load and unload its oxygen at suitable oxygen tensions, and *c*, be able to do it quickly.

a. The storage capacity must be measured in terms of the oxygen requirements. As a typical example of a red muscle let us take the normal dog's heart. Its MHb concentration is about 0.5 per cent, the oxygen capacity of which is about 0.008 cc. per gram of tissue, while the oxygen consumption is about 4.5 cc. per gram per hour; the entire

stored oxygen would be used up 4.5/0.008 or 560 times an hour or once in about 7 seconds. In extreme activity, the metabolic demands may be ten times this amount, when the oxygen store would last only about half a second, which is just about sufficient to tide the muscle over from one contraction to the next. The store must therefore be a short time one.

If the output of the organ were to remain about the same but the frequency of contraction were to increase, the storage demands would diminish until ultimately the oxygen in solution might satisfy them. This would account for the absence of the pigment in the rapidly-beating hearts and in wing muscles of insects, and would account for its presence in slow-beating hearts, in the diaphragm, and in running muscles requiring maintained repetitive activity. An exceptionally high concentration occurs in the muscles of the seal, where according to Theorell (44) the muscle press juice may contain from 5 to 10 per cent of pigment. This is something like ten times the concentration normally found in mammals, and should provide an oxygen supply sufficient to last for half a minute or so of moderate activity. In this animal, then, the store function may have been extended to far longer periods than that between one contraction and the next. This fits in well with the animal's known ability to swim for some time under water.

b. The affinity of MHb for oxygen lies between that of blood Hb and the oxidases, so that it is well adapted to take up oxygen from the one and give it out to the other. This may be seen from the dissociation curves of figure 1, which has been taken from the paper by R. Hill (1936), extended from Barcroft. At a venous pressure of 40 mm. Hb is 66 per cent saturated; the MHb at this tension is 94 per cent saturated, so that even at the venous end of the capillaries the store will be nearly full, though the blood supplies are seriously depleted. But the store to be of any use must be emptied as well as filled, i.e., the cells must be able to take up oxygen at an oxygen pressure so low that even MHb has given up a considerable fraction of its oxygen. This is just what Warburg and Kubowitz have found them capable of doing (45). They found for yeast at 10° that 65 per cent of full aerobic respiration remained at a pressure of under 0.5 mm. Hg, and it is probable that even here the limiting factor was not the affinity of oxidase for oxygen but diffusion within the cell. Experiments by Keilin (19) on heart muscle preparations indicate that for this tissue also the oxidase can function at pressures below 5 mm. It can be seen from figure 1 that at this

pressure the pigment has already given up 40 per cent of its total store, while at 1 mm. pressure it has yielded up over 65 per cent.

c. The rates of reaction with oxygen are rapid enough to enable MHb to load and unload very rapidly, the times for half reaction being of the order of 0.001 and 0.01 second respectively under physiological conditions.

Direct observation of the store function in vivo. It is possible to see and record the use of muscle hemoglobin as a store in the soleus muscle

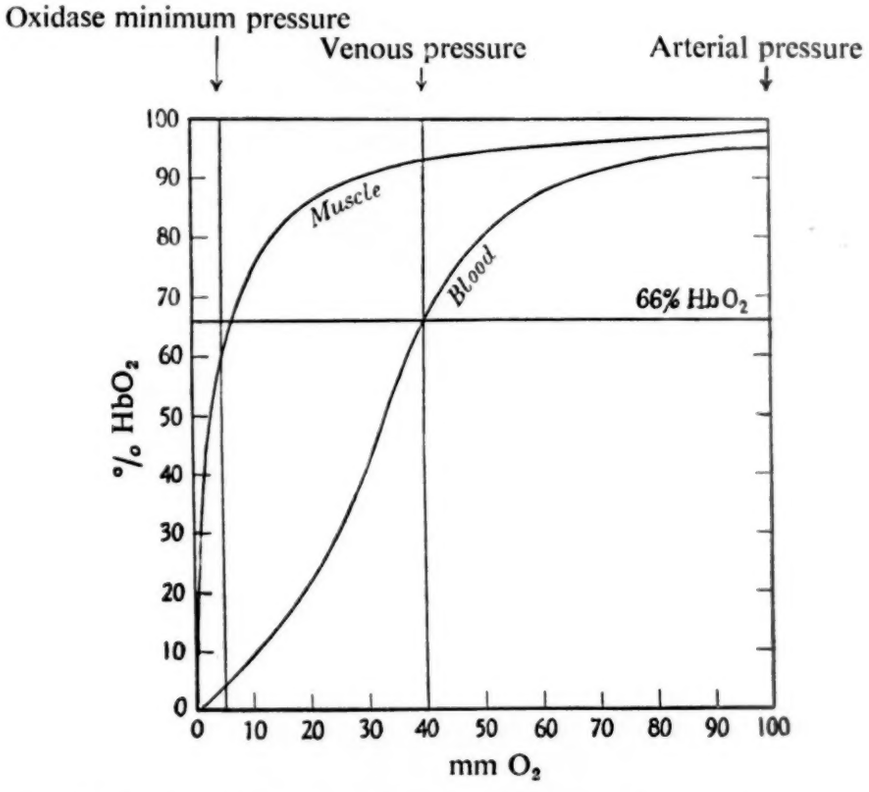

Fig. 1. The relationships of the dissociation curves of muscle hemoglobin and of blood to the minimum oxygen pressure required by the tissue. (R. H.: 15, 1936) (reproduced by permission).

of the cat, unexcised and with the nerve and blood supply intact (25). In these experiments light was passed through the muscle transversely and the colour of the emergent beam analyzed by means of a differential photocell colorimeter, by means of which the degree of oxygenation of the MHb could be continuously recorded, the galvanometer deflections of the colorimeter being photographed on moving bromide paper. When the muscle was tetanized, it instantly began to change its colour, the MHb losing from 9 to 40 per cent of its oxygen in the first second of

contraction, even though the blood supply was left intact. Thus in the normal, intact muscle the blood was unable to supply the instantaneous oxygen demands of the tissue during contraction, and the store was drawn upon. As soon as the muscle was allowed to relax, it began to regain its redness, the store being replenished. A tracing of a typical record is reproduced in figure 2.

A store can operate either to smoothe out a fluctuating supply, or it can enable the organ possessing it to make fluctuating demands under conditions of steady supply. Muscle hemoglobin appears to act primarily in the second of these two ways, at any rate in the soleus of the

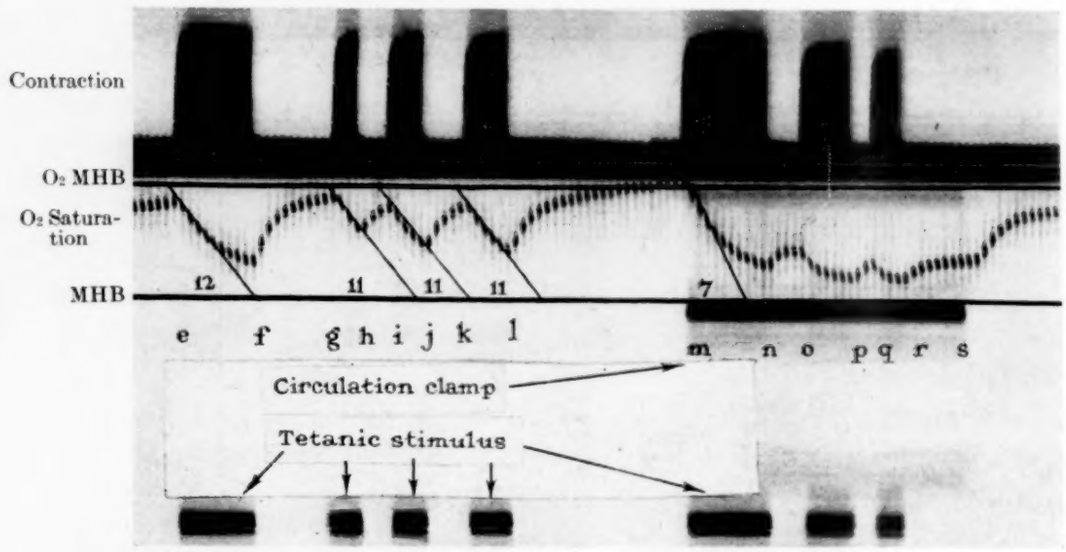

Fig. 2. Oxygen storage by muscle hemoglobin in the soleus of the cat. At the left the circulation is intact, at the right it is clamped off in the descending aorta. The interruptions in the oxygen saturation curve are 1 second time marks. (G. M. 25 reproduced by permission.)

cat. In this preparation, maximum contraction of the muscle showed little tendency to reduce the flow through it, as could be seen by applying an external clamp on the supplying artery, and observing the much more rapid oxygen depletion (right hand side of fig. 2). It is not the purpose of this review to enter into the much-debated question of blood flow through muscle during contraction, though the very recent work of H. Barcroft on the intact human gastrocnemius under voluntary sustained contraction provides convincing evidence that a stoppage under some circumstances does take place. In such muscles the store acts in both ways, for the supply fails just as the need is greatest, and we have both intermittent supply and intermittent

demand. The value of the store depends upon the speed with which oxygen is demanded when the muscle contracts. This will be considered in the next section.

5. Rapid chemical changes in muscle. *The instantaneous measurement of oxygen consumption.* Quite apart from its physiological usefulness, muscle hemoglobin has the unique distinction of being a naturally occurring intracellular oxygen tension indicator with a very short time of response (about 1/100 sec.) Three properties fit it for this rôle: *a*, its colour change on oxygenation, enabling optical methods of measurement to be used, which involve no damage to the cell; *b*, its working range from a fraction of a millimeter of oxygen tension up to 20 or 25 mm., which is the working range through which the muscle itself operates;[6] *c*, its ability to load and unload quickly, thus bringing it into rapid equilibrium with its oxygen environment. These put a new tool into our hands, for they enable us for the first time to follow the detailed time course of oxygen utilization inside a living cell, during normal and extraordinary metabolic activity. Previously available *time-sensitive* methods of studying cell metabolism (electrical, thermal, morphological, etc.) have not been *chemically-specific:* the measured quantities have been the algebraic sum of several phenomena. Manometric and gas exchange methods, on the other hand, require several seconds at the very least for equilibration, the speed being limited by diffusion processes in the cells, through the bathing medium and across phase boundaries. Sudden freezing and subsequent analysis involves irreversible destruction of the tissue. The MHb method is specific for oxygen, but it is also rapid.

Using this "metabolic movie camera," (25) we find that in the cat's soleus increased oxygen consumption gets under way in less than $\frac{1}{5}$ of a second after the start of contraction, and has reached its maximum rate before 1 second has elapsed, these figures being the minimum response time and the minimum measurement time of the instrument respectively, so the times may be much shorter even than this. The rapid uptake can be seen from the abrupt dive of the oxygen curve at the onset of each contraction in figure 2. This "quick oxygen," furthermore, constitutes the great bulk of the total oxygen required by the contracting muscle; this is deduced from the fact that the rate of

[6] The high oxygen affinity of MHb has also been made use of by R. Hill (16) for detecting small amounts of oxygen produced by isolated chloroplasts undergoing photosynthesis.

oxygen consumption increased tenfold during contraction, but returned nearly to its resting value ten seconds after the end of tetanus.

Implications for muscle metabolism. There are two ways in which we might explain the rapid oxygen consumption. The first is by means of the classical theory, according to which the anaerobic contraction process is followed by oxidative recovery and restitution; the second is by postulating a direct oxidative mechanism in addition to the anaerobic one. It is not easy to reconcile the slow aerobic recovery heat (on the classical theory this is the time during which the oxygen is consumed) with the very rapid rate of oxygen uptake found in the MHb experiments. In frog sartorius muscle at low temperatures the recovery heat is only half liberated in ten minutes, and very recent work of D. K. Hill (13a) has shown that under these conditions the oxygen uptake runs parallel to the recovery heat production. The difficulty is not necessarily an insuperable one, however, for frog muscle at 0° and mammalian muscle at 37° are very different tissues, and an increase in reaction rates of several hundred fold is quite conceivable. Cattell and Shorr (7a) report that in dog's scalenus muscle at 37°, the recovery heat is very rapid, reaching its maximum rate in a few seconds, and could only be separated from the initial heat by the method of curve analysis. Is it possible that if we could replace isolated portions of scalenus muscle by uninjured muscle with circulation intact, the few seconds would be reduced to a fraction of a second?

More difficult to explain away is the direct chemical evidence against the classical view, which has been accumulated in the last few years by Sacks and Sacks. The recent paper by Sacks (36) summarizes their present position. They have studied the recovery rates of the three chemical processes which have been associated by biochemists with anaerobic muscular activity, after a "steady state" period of stimulation. The results are comparable with those of the MHb experiments, for they were obtained on the gastrocnemius muscle of the cat, which had been contracting under relatively normal conditions until the moment of freezing of the tissue for chemical analysis. The three reactions studied (the removal of lactic acid, the resynthesis of phosphocreatine, and the resynthesis of adenosine triphosphate) were each of them found to require several minutes, and were therefore much too slow to play a significant rôle in rapid oxidative recovery. The adenosine triphosphate resynthesis was somewhat less satisfactory than that of the other two reactions, as the changes measured were very much smaller. The acceptance of these results does not necessarily mean the

abandonment of the classical theory of anaerobic contraction and oxidative recovery. It does, however, mean something nearly as catastrophic, namely, that none of the reactions of anaerobic muscle biochemistry seem to be of much importance for aerobic activity.

The theory that a direct oxidative process provides the energy for contraction was proposed by Sacks in 1932. Since the ability of muscles to operate for a time anaerobically is unquestioned, this hypothesis requires a dual mechanism: one, a normal, aerobic, quick, efficient mechanism; the other, an emergency, oxygen debt mechanism, presumably of lower efficiency, called into action when there is an oxygen lack. In terms of the well-known analogy, the muscle can run off its batteries if it has to, charging them up afterwards, but it prefers to operate by direct drive if it can.

This is, in a way, a negative theory, and it is inherently improbable. It attacks—though not for the first time—all of the three chemical reactions proposed by muscle biochemistry as the principal energy-producing processes of aerobic contraction, but it proposes no alternative chain. For "direct" we might as well read "unknown but rapid." And it is unlikely, for striated muscle gives no hint of this dual nature in its histological structure or in its chemical composition. If two mechanisms have developed side by side, is it not strange that throughout the animal kingdom we have not a single example of a muscle in which the inefficient anaerobic process has been allowed to degenerate?

But neither of these objections should be taken too seriously. They are philosophic rather than scientific, and are merely a reflection of our lack of knowledge. They but emphasize the fact that the fundamental problem of muscular physiology, the mechanism of transfer of energy from the chemical to the mechanical system, is still quite unsolved. The relation of aerobic to anaerobic metabolism will be an inherent part of the solution to that problem.

SUMMARY

Muscle hemoglobin occurs in those muscles requiring vigorous repetitive activity, which must be maintained. Its concentration increases with age and activity. It can be obtained in crystalline form, is a true hemoglobin, but differs from blood hemoglobin quantitatively in the following ways: 1, its absorption bands are displaced to the red; 2, it has a very high oxygen affinity, and a hyperbolic dissociation curve; 3, it has only one iron atom per molecule; 4, it has a very small Bohr effect; 5, it has a low affinity for CO relative to O_2.

There is no evidence that muscle hemoglobin acts catalytically, though its properties are intermediate between those of the blood hemoglobins and oxidation enzymes. It does act as a short-time oxygen store, tiding the muscle over from one contraction to the next.

As an indicator of oxygen tension in the cell, muscle hemoglobin enables us to follow the time course of oxygen uptake during rest, contraction, and recovery. It is found that oxygen demand rises instantaneously during contraction, the bulk of all the oxygen needed being utilized in less than one second from the time the work is performed.

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THE ARCHITECTURE OF VIRUSES

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The atomic theory of matter, the germ theory of disease, and the cell theory of life may appear at first to be quite diverse and unrelated. However, it will be the purpose of this review, not only to present the recent advances in virus research, but also to indicate that the successful continuation of this work may be dependent upon an intimate knowledge of the three theories just mentioned and even, perhaps, upon their integration into a new and unified philosophy. The atomic theory, probably first stated before 1000 B.C., has been subjected to continual modification in detail as a more exact knowledge of atomic structure has been gained, yet the original and basic concept of matter as a discontinuous rather than as a continuous or homogeneous phase has remained unaltered. The germ and cell theories have been subjected to less modification than has the atomic theory, perhaps because of their more recent origin, hence with time and new knowledge it would not be unexpected if they too should require further alteration. It is an unfortunate fact that knowledge gained before its due time frequently results in a strong desire to overthrow useful theories, and it is only later, following understanding, that reconciliation and accord are achieved. For example, it is known that many present day facts are entirely inconsistent with the atomic theory as stated by Dalton in 1808, yet the evolution of the theory has been such as to account for these facts and to provide today a valid and coherent atomic theory. The newer knowledge of viruses may appear inconsistent with present day theories, yet there is every reason to believe that a more complete understanding of the structure or architecture of viruses will permit the blending of hypotheses and facts into a unified philosophy without the overthrow of time-honored theories.

The word "virus" was originally used only in the singular and meant a poison such as a snake venom. Later it was used to denote infectious disease-producing entities without regard to their nature, and more recently it has been applied only to those infectious agents capable of

passing through filters that retain ordinary bacteria. Today, properties ascribed to viruses include not only their ability to pass fine membranes but also a set of general properties which emphasize the intimate relationship that exists between viruses and their host cells. These include the fact that viruses reproduce, but do so only within certain living cells, the fact that during reproduction they may change or mutate, the fact that many virus-infected cells contain inclusion bodies, and the fact that most, but not all, virus diseases are followed by a lasting immunity in recovered hosts. Tobacco mosaic, which was the first of the agents now known as viruses to be discovered, was shown to be filterable by Iwanowski (1) only 47 years ago and was recognized as a new kind of infectious agent by Beijerinck (2) 41 years ago. Other viruses to be recognized since then include those responsible for the foot-and-mouth disease of cattle, louping ill of sheep, hog cholera, rabies, dog distemper, fowl pox, smallpox, psittacosis, yellow fever, St. Louis encephalitis, horse encephalitis, poliomyelitis, fever blisters, certain types of tumorous growths in fowls and other animals, various yellows and mosaic diseases of plants, and even the unusual colors produced in the flowers of plants which in tulips is called tulip break. Still more viruses are being discovered from time to time.

It should be emphasized that viruses were first recognized and have continued to be recognized only by means of their biological activity, that is to say, by the diseases that they cause. They were regarded merely as infectious disease-producing principles and, with the exception of the elementary bodies of vaccine and other viruses which are as large as accepted organisms, attempts to isolate a virus in tangible form had resulted in failure, hence little or nothing was known of their true nature. An element of mystery tended to surround them, and they were regarded variously as invisible forms of ordinary bacteria, as protozoa, as some new type of invisible living organism, as enzymes, as unusual products of cellular metabolism, as toxins, and as different kinds of chemical principles. However, in 1935 it was found possible to isolate from mosaic-diseased plants by chemical means a material which appeared to be a high molecular weight protein and which was distinguished by the fact that it possessed the properties of tobacco mosaic virus (3). This was followed by the isolation, by the same or similar chemical methods or by means of differential centrifugation, of similar high molecular weight nucleoproteins possessing the properties respectively of aucuba mosaic (4), enation mosaic (5), tobacco ring spot (6), latent mosaic of potato (6–8), severe etch (6), Shope rabbit papil-

loma (9), bushy stunt of tomato (10), cucumber mosaic 3 and 4 (11), and tobacco necrosis (11a, 11b) viruses, and of a staphylococcus bacteriophage (12). The presence of high molecular weight protein material in very active preparations of chicken tumor I (13), equine encephalitis (14), and foot-and-mouth disease viruses (15) has also been demonstrated, although Claude's (16) isolation of a fraction from normal chick embryo that is quite similar to the purified chicken tumor I preparation casts some doubt upon the significance of the results in the cases of the first two viruses. The isolation of the different high molecular weight nucleoproteins was of importance because at last tangible materials possessing quite definite physical and chemical properties were available for study, and thus the possibility was offered of correlating virus activity with such properties. Although some of the mystery surrounding viruses was removed by the isolation of the nucleoproteins carrying virus activity, the isolation really represented but a small step towards the solution of the problem of the ultimate nature of viruses. Protein structure may be expressed in many different ways as in hormones, enzymes, toxins, respiratory materials, and perhaps as in genes and as in protoplasm, and, since practically nothing is known about protein structure, the addition of viruses to this diverse group aided but little in the establishment of their true nature. All viruses appear to have a high molecular or particle weight, yet this fact alone can not be used as a criterion of virus activity (17, 18), for some of the virus nucleoproteins may be inactivated by appropriate treatment without changing their size greatly. However, no entity having a size smaller than that corresponding to a molecular weight of about 400,000 has been found to possess virus activity, hence it is probable that a certain amount of structure may be necessary to support such biological activity.

At the present time the solution of the virus problem appears to reside in the elucidation of the structure peculiar to materials carrying virus activity. This problem is more complex than that involved in protein structure as such, for, of the virus materials so far isolated, none have been found to yield only amino acids on hydrolysis. The simplest appear to be composed of protein plus nucleic acid, the more complex of protein, nucleic acid, and carbohydrate, and the most complex of materials indistinguishable from those found in bacteria. These results may be interpreted as indicating that viruses are protein or protein-like in nature, but there is little justification for the tendency to accept the results as demonstrating unequivocally that viruses are non-living

and are merely ordinary protein molecules similar to egg albumin. Although the physical and chemical properties of the virus nucleoproteins are similar to those of ordinary proteins, the biological properties are quite different, and it is because of the virus activity, which implies the ability to multiply and to change or mutate, that it is difficult to conclude that they are ordinary protein molecules. Despite this difficulty, the virus nucleoproteins which have been found to have the chemical and physical properties of molecules will be referred to as molecules in this review. Others may wish to refer to these same particles as organisms or cells, yet, as will be indicated in the last section, the question of nomenclature is of secondary importance. The virus activity is undoubtedly a consequence of the unique architecture of the materials that have been isolated, yet it is necessary, before proceeding with such an assumption, to ascertain whether or not it can be demonstrated that the virus preparations are essentially pure and that the virus activity is a specific property of the major component. This demonstration is dependent upon the correlation of chemical and physical properties with activity, hence virus activity and its measurement become all-important considerations and will, therefore, be discussed first.

Virus activity—its measurement. The most characteristic and at the same time the most important property of viruses is their biological activity, their infectiousness or ability to multiply or reproduce when introduced into certain living cells. It is this property that resulted in the original discovery of viruses, and it is this property, more than any other, that has caused viruses to be regarded as elementary living organisms, for the ability to multiply within an essentially non-specific and variable environment has been generally considered to be one of the essential characteristics of living organisms. This property also distinguishes viruses from ordinary protein molecules, such as those of egg albumin and of hemoglobin, or even from the biologically active enzyme proteins, such as pepsin or trypsin which have the ability of multiplication in that they can cause the autocatalytic conversion of specific inactive precursors to pepsin and trypsin. It may be noted here that, should specific virus precursors be found, the virus reaction would then resemble that of the enzymes, pepsin and trypsin.

During most of the early work with viruses, no attempt was made to measure virus activity quantitatively. The activity determinations were merely qualitative and were made to determine whether or not a given preparation could cause infection. In these tests, a number of

animals or plants were inoculated and the appearance of disease symptoms was used as an indication of the presence and transfer of virus. In most of these tests, one animal or plant was required for each successful transfer of virus and it was not known whether one or more than one infective doses of virus had been administered. Later, in an effort to secure a quantitative measure of the amount of virus present in the preparation, a number of animals or plants were inoculated with various dilutions of the preparation, some of which were sufficiently dilute so that no infection followed the inoculation. This procedure established minimal infective doses and permitted the estimation of the number of such doses in the original virus preparation. However, this method of estimating virus quantitatively was subject to great error due to the tremendous variation in the response of the test animal or plant. Although by careful standardization the method can be made reasonably accurate, in most cases no attempt was made to estimate differences in virus concentration less than that corresponding to a 10-fold difference, viz., one power of ten. It is obvious that in the isolation and fractionation of virus preparations there could be differences of several hundred per cent in the virus content of different samples that would go unrecognized. The fact that it was difficult, and in many instances impossible, to follow the major portion of the virus in preparative or fractionation work was, perhaps, one of the severest handicaps that was imposed upon the earlier workers.

Calmette and Guérin (19) in 1901 noted that the number of lesions produced on a rabbit following inoculation of vaccine virus could be used as a measure of the potency of the virus preparation, but it was not until the years immediately following 1929, when Holmes (20) reported similar results with tobacco mosaic virus on certain plants, that the great usefulness and accuracy of the local lesion method of virus estimation was demonstrated. Since each primary lesion is indicative of the successful transfer of virus, and since it is possible to secure many lesions on a single animal and literally hundreds of lesions on a single leaf, it is obvious that one individual giving such a response can yield information obtainable only with hundreds of individuals giving a systemic response. The utilization of this fact has been of signal importance in plant, as well as in animal, virus research. The local lesion method of Holmes has been modified by Samuel and Bald (21) and by Youden and Beale (22) and standardized by Loring (23), so that today there is a method available for the estimation of the concentration of tobacco mosaic virus that is sufficiently accurate so that differences in

virus concentration of 10 per cent or greater may be detected without undue effort. Similar methods are used by Rous and co-workers (24) in the titration of the Shope rabbit papilloma virus, although the accuracy is probably not as great as that achieved with tobacco mosaic virus. It should be emphasized that the methods under discussion are for the estimation of the relative amounts of virus present and not for the estimation of absolute amounts of virus. Although Parker and Rivers (25) have used a dilution end-point method in estimating vaccine virus with success, in most cases attempts to estimate the absolute amount of virus present in a sample by the titration end-point have yielded very erratic results. In the case of tobacco mosaic virus, a single preparation may yield dilution end-points differing by a factor of a thousand or more, depending upon the conditions and the test plant that are used. Even when tests are carried out at the same time and with the same batch of test plants, 10-fold differences in the dilution end-point are encountered.

It was hoped that with the isolation of tangible materials carrying virus activity it would be possible to inoculate solutions containing given amounts of such materials and secure definite numbers of lesions. Although the order of magnitude of the number of lesions given by a definite amount of virus is known, viz., a solution containing 10^{-5} grams of carefully prepared tobacco mosaic virus nucleoprotein per cubic centimeter in 0.1 M phosphate buffer at pH 7 will usually yield from 50 to 150 lesions per leaf on *Nicotiana glutinosa* L., the method can not be used as an absolute measure because of the variation in the number of lesions obtained. Under adverse conditions, such a sample may give in the neighborhood of 50 lesions per leaf, whereas under especially good conditions, 150 or more lesions may be obtained. Since a similar variation in the number of lesions may result from differences of 100 per cent or more in the actual concentration of virus, it is impossible to be certain from the absolute number of lesions obtained whether any variation is due to a difference in the amount of virus or to the conditions under which the test is carried out. There is today, therefore, no way of estimating directly and accurately absolute amounts of tobacco mosaic virus by activity measurements. It is necessary to estimate the virus by comparing the activity against that of an arbitrarily selected standard under well defined conditions. Since it is known that differences in virus activity of 10 per cent or more may be readily detected, the sole difficulty in the measurement is the selection of the standard used for comparison. Many different viruses are known to lose activity rapidly

when allowed to stand, and recent evidence indicates that even the supposedly very stable tobacco mosaic virus is actually unstable in that a loss in activity can be demonstrated if the virus is allowed to stand over a sufficiently long time. It is obvious, therefore, that at best a preparation gradually changing in activity must be used as a standard. This is not a serious difficulty in the case of viruses as stable as that of tobacco mosaic, for a period of several weeks is required before the change can be detected. A more serious and practical difficulty is the fact that different methods of preparation yield samples that differ widely in activity. Since some methods of preparation obviously cause the inactivation of much virus and yield preparations of low specific activity, the most active virus preparation obtainable should be used as the ultimate standard for comparison. In the case of tobacco mosaic virus, it has been found that a few careful ultracentrifugations of the untreated juice from frozen badly diseased Turkish tobacco plants yield preparations which do not differ measurably from each other in activity and which on a protein basis possess activity that is not measurably different from that of the virus in the untreated juice (26). Such preparations possessing this maximum activity are referred to as "fully active" virus and should be used as the standard for comparison.

In the early work with tobacco mosaic virus, some preparations were obtained by subjecting the virus to strong concentrations of ammonium sulfate at room temperature and, because the methods of measuring virus activity then in use were not sensitive, it was not found that such preparations varied widely in activity depending upon the extent and nature of the chemical treatment. Recent work has demonstrated, however, that such treatment may yield preparations possessing only 1 per cent of the original activity. Such almost completely inactivated preparations may still be crystallized, will yield liquid crystalline solutions, and in many other respects are quite similar to fully active preparations. Whether such preparations consist of a mixture of a small amount of fully active virus plus a large amount of completely inactive virus, or of an essentially homogeneous phase of units, each of which possesses a greatly lowered activity, is not known, but it seems likely that the former is the case. The failure to recognize the difference between partially active and fully active preparations has resulted in many difficulties and misunderstandings, and it is to be hoped that the experience with tobacco mosaic virus will aid in the research with other viruses, where in most cases the situation will be even more difficult. For example, Loring (27) has found that merely allowing latent

mosaic virus to stand in the presence of 10 per cent ammonium sulfate for one day at room temperature causes a 50 per cent reduction in the number of lesions given by the sample, and that isolation of the virus from juice by means of 5 precipitations with salt yields a preparation having only about 10 per cent of the activity of a preparation obtained from the same juice by ultracentrifugation. Similarly, the writer has found that one precipitation of tobacco ring spot virus with 30 per cent ammonium sulfate results in a loss of about 50 per cent of the activity, and that solutions of this virus in water lose activity rather rapidly, even when held at 2°C., whereas solutions in 0.01 M phosphate buffer at pH 7 retain their activity fairly well, provided they are held at 2°C. However, even with these rather unstable viruses it has been found possible to obtain preparations having a high and uniform activity and probably consisting, therefore, of fully active virus. It may be concluded that, while it is not possible to estimate the absolute amount of virus present in a sample solely by means of its activity, methods are available for detecting small differences in the concentration of those viruses giving a local lesion response. In such cases it is possible not only to differentiate between preparations of low and high activity, but also to establish, as the standard for comparison, a preparation which may be referred to as fully active virus.

Correlation of virus activity with chemical and physical properties. The demonstration, beyond a reasonable doubt, that the virus preparations which have been isolated, or at least the major component in each of the preparations, actually consists of virus is of paramount importance, for the further consideration and the acceptance of the preparations as virus are directly dependent upon such demonstration. Although much of the work done since the isolation of the purified virus preparations has been devoted to a study of this problem, there will be presented here briefly only the more significant results, most of which have been obtained with tobacco mosaic virus. This virus has one of the widest host ranges known, for 46 different species of plants representing 14 widely separated families are susceptible to the mosaic disease (28). Although attempts to obtain purified preparations from all of these have not been made as yet, virus preparations possessing essentially the same chemical, physical, and biological properties have been obtained from different batches of diseased Turkish tobacco, Burley tobacco, tomato, phlox, spinach, petunia, and nightshade plants. However, the yield of virus obtained was found to vary widely depending upon the host. Turkish tobacco plants gave a yield

of 2 to 5 mgm. of purified virus per cubic centimeter of juice, tomato plants about 1 to 3 mgm. per cubic centimeter, and spinach and phlox plants considerably less than 1 mgm. per cubic centimeter. It should be noted that some of these plants, such as phlox and spinach, are far removed from the tobacco family. For example, no serological relationship was found by Chester (29) between the protein from normal tobacco plants and that from normal phlox plants. The fact that on infection these two different plants foster the production of the same virus is an indication that virus is not produced by the simple polymerization of serologically active normal proteins.

There is good evidence that during production in a host viruses occasionally change or mutate and give rise to new strains which may be isolated, grown, and studied apart from the parent virus (30, 31, 32). Many strains of tobacco mosaic virus are recognized and to date 4 of these have been isolated in purified form. Preparations of strains of the same virus have been found to have somewhat similar general properties, yet it was found possible to distinguish each of the 4 preparations by means of definite and characteristic chemical, physical, and serological properties (4, 5). Furthermore, the concentration reached in a given host was found to vary widely with the strain of virus (33). The amounts of the different purified viruses obtainable from a given quantity of starting material have also been found to vary widely. For example, some batches of badly diseased Turkish tobacco plants have been found to contain one part of virus per 200 parts of fresh green plant material, whereas Turkish tobacco plants diseased with cucumber mosaic 1 virus contain only about one part per million as virus. Beard and Wyckoff (9) obtained about one part per 5000 of the starting material, based on the whole rabbit, in the form of the papilloma virus, and Northrop (12) estimated that the crude culture of the staphylococcus with which he worked contained about one part per million as bacteriophage. It should be recognized, therefore, that the amount of virus occurring in a host may vary tremendously depending upon the virus and the host, some viruses occurring in amounts as great as a part per 200 and others in amounts as small as a part per million, with the possibility that other viruses may occur in still smaller amounts. In those cases where it has been found impossible to isolate weighable amounts of virus, or to demonstrate the presence of virus by immunological reactions, it is possible that the virus was extremely unstable or existed in such great dilution that it escaped detection by means other than activity measurements. The importance of considering

antigenic mass in virus immunization studies and in immunological reactions *in vitro* has been emphasized by Merrill (34). The purified preparations of the various viruses, such as latent mosaic, tobacco ring spot, bushy stunt of tomato, and the Shope rabbit papilloma viruses, and a staphylococcus bacteriophage, have been found to have quite different and highly characteristic chemical, physical, and serological properties. It may be concluded that the different viruses as well as the various strains of a given virus reach widely different concentrations in their hosts and that the respective purified preparations possess definite and highly characteristic chemical, physical, and serological properties which may be correlated with the biological or virus activity.

The purified viruses that have been isolated have been found to consist largely of protein which is susceptible to digestion with certain proteolytic enzymes and which may be denatured by appropriate treatment. In every instance so far studied, the digestion or denaturation of the protein has been accompanied by loss of virus activity and, in general, the rate of digestion or of denaturation and the rate of loss of activity have paralleled each other. The studies have included denaturation by acid, alkali, heat, dodecyl sulfate, urea, etc. It should be noted here that the purified preparations of the different viruses were found to be stable only over certain ranges of hydrogen ion concentration that were definite and characteristic for each virus. In most instances, at the same pH that caused loss of activity, there occurred a break-up of high molecular weight material into low molecular weight material that could be demonstrated by means of the analytical ultracentrifuge. It has also been found possible to inactivate some virus preparations, such as those of tobacco mosaic, cucumber mosaics 3 and 4, and latent mosaic, with nitrous acid, hydrogen peroxide, formaldehyde, or ultraviolet light without the accompanying gross change in properties that is usually referred to as denaturation. The general chemical, physical, and serological properties of the inactivated preparations are very similar to those of active preparations. For example, in the case of tobacco mosaic the preparation still consists of a high molecular weight nucleoprotein which may be crystallized and which reacts specifically with antiserum to active virus. Should it prove possible to inactivate a virus with but a very slight change in its makeup, it is possible that very sensitive chemical or physical tests might be necessary in order to detect the change. However, despite the great similarity in general properties, in every case yet studied it has been possible to demonstrate that the inactivation was accompanied by a

measurable change in one or more of the chemical, physical, or serological properties of the preparation. In the treatment of tobacco mosaic virus with formaldehyde, the inactivation was accompanied by a decrease in amino nitrogen as measured colorimetrically or by means of the Van Slyke gasometric method and by a decrease in the color developed by Folin's phenol reagent (35). Of considerable importance in connection with the correlation of chemical with biological properties are the facts that it was found possible to reactivate the formolized virus and to demonstrate that the reactivation was accompanied by an increase in amino nitrogen, as measured by the color developed with ninhydrin, and also by an increase in the color developed with the phenol reagent. This correlation indicates that the virus activity is a property of the nucleoprotein and provides information concerning the structure necessary for activity.

The action of 36 per cent urea in 0.1 M phosphate buffer at pH 7 on tobacco mosaic virus has been studied by Frampton and Saum (36) and at different hydrogen ion and salt concentrations by the writer with Lauffer (37), and the action on the same virus of 0.5 per cent or greater concentrations of sodium dodecyl sulfate at pH 8 was studied by Sreenivasaya and Pirie (38). In the two latter studies, the virus was found to be rapidly disintegrated into low molecular weight protein components free of nucleic acid with loss of virus activity, serological specificity, and ability to show stream double refraction. Frampton and Saum reported that, although low molecular weight material was found, there was no change in virus activity, a result that appears very questionable and one which it has not been found possible to confirm in the writer's laboratory. In the case of treatment with urea, the denaturation was accompanied by the appearance of sulfhydryl groups as indicated by a positive nitroprusside reaction, and the low molecular weight protein which was formed was soluble in water but insoluble in 0.1 M or more concentrated salt solution.

The fact that the virus preparations that have been isolated have consisted essentially of high molecular weight protein material has been utilized in still another type of experimental approach. The materials can be sedimented from solution by means of a high speed centrifuge because of their unusually high molecular weight, and, since they are protein and have an isoelectric point, the sedimentation of negatively charged protein, of positively charged protein, and of neutral or uncharged protein can be studied. In the case of tobacco mosaic virus, it was found that, regardless of the charge, the protein and the

agent carrying the virus activity sedimented at exactly the same rate. This is good evidence that the activity is a property of the protein, for, if the activity were due to a small amount of some material of a different size mixed with the protein, it is obvious that under one of the conditions cited the mixture would have been separated and the activity would not have been found with the high molecular weight protein. It was found possible to separate, by the high speed centrifugation of mixtures of tobacco mosaic virus with egg albumin, globin, trypsin, or pepsin, the characteristic high molecular weight nucleoprotein with unchanged activity (39). Gratia and Manil (40) obtained similar results with mixtures of tobacco mosaic virus and a bacteriophage.

Neurath and Saum (41), using the refractometric method of Lamm, found the diffusion constant of chemically purified tobacco mosaic virus to be about 3×10^{-8} cm.^{2}/second, a value corresponding to a molecular weight of about 50 million. The diffusion constant obtained by Hills and Vinson (42) by means of the Northrop-Anson diffusion cell is probably far too large, since these workers did not use sufficient electrolyte to eliminate the accelerating effect on the virus of small ions. In most of their experiments, virus was permitted to diffuse from a dilute electrolyte solution into distilled water, and in the remaining experiments, from a trypsin solution into a trypsin-free solution. Purified preparations of tobacco mosaic virus have been subjected to a careful immunological study, and when sufficiently purified it was not found possible to detect material other than the virus protein even by the sensitive precipitin and anaphylactic tests (43). Evidence of a different nature but also indicative of unusual homogeneity was provided by the results obtained by means of the analytical ultracentrifuge and the electrophoresis apparatus, for in each case a single sharp boundary characteristic of a single molecular species was obtained (44, 45). It was found impossible to separate virus activity from protein by filtration through collodion or other types of filters. The ultraviolet light absorption spectrum of purified tobacco mosaic virus preparations was found to agree essentially with the destruction spectrum of virus activity, thus indicating a close relationship between the two (46, 47).

Northrop (12) has amassed an impressive amount of experimental evidence which indicates that phage activity is a property of the high molecular weight protein isolated from lysed staphylococcus cultures. He found the protein diffused across a sintered glass membrane and sedimented in the ultracentrifuge at exactly the same rate as did the active agent. Of interest is the fact that evidence was secured which

indicates that the phage particles are considerably larger in concentrated solution than in dilute solution. The partial denaturation of phage protein preparations, by acid, alkali, heat, or enzymes, was accompanied by a corresponding loss of phage activity. The absorption spectrum of the phage protein was found to agree essentially with the destruction spectrum of phage activity. Of special significance is the fact that Northrop found the solubility of two especially active preparations of phage protein to vary only slightly with a change in the amount of solid phage present, a result indicative of unusual homogeneity.

A great amount of experimental work on the purified virus preparations has already been completed, and an even larger volume is now in progress. Still more viruses are being obtained in purified form from time to time and ever-increasing amounts of the ones already purified are being made available for experimentation. In all of the work that has been reported to the present time, or that is known to the writer, not a single bit of experimental evidence has been obtained that is incompatible with the idea that the various purified and unaltered virus preparations, or at least the chief component, actually consists of the active agent. On the contrary, there is an imposing array of evidence which indicates that several of the viruses have been isolated in an essentially pure form and that the biological activity is a specific property of the respective nucleoproteins. There is always the possibility that the virus materials may represent a unique situation and that there is something which has not been comprehended as yet. However, it seems to the writer that the time has arrived, or at least is rapidly approaching, when it can be said that virus activity is a specific property of the nucleoproteins, with the same degree of assurance as when it is said that the properties of water are those of the water molecule or that the hormone properties of insulin belong to the protein molecule known as insulin. On the basis of information now available, it must be concluded, therefore, that the different nucleoproteins represent the respective viruses in essentially pure form. The composition and structure of the purified materials become of importance, for if virus activity is a specific property it must result from a characteristic architecture. The information now available on the composition of the purified viruses will be considered in the next section.

Composition. Quantitative chemical analyses have been made on purified preparations of tobacco mosaic virus and its strains aucuba and enation mosaics, the related viruses cucumber mosaics 3 and 4, tobacco ring spot, latent mosaic of potato, bushy stunt of tomato, chicken tumor

I, and vaccine viruses and on a purified preparation of a staphylococcus bacteriophage. The values for carbon, hydrogen, and nitrogen for the plant virus preparations were about 50 per cent, about 7.5 per cent, and about 16 per cent, respectively. The phage preparation was found to contain about 41 per cent carbon, 5.3 per cent hydrogen, and 14.4 per cent nitrogen. The vaccine virus preparation contained 13.4 per cent nitrogen and 6.5 to 10.1 per cent lipoid and the chicken tumor I virus preparation only 8.6 to 9.5 per cent nitrogen and 24 per cent lipoid. Perhaps the most interesting of the analytical results are those reported for sulfur and phosphorus, for these have been found to vary considerably from virus to virus. Preparations of tobacco mosaic virus contained 0.24 per cent sulfur and 0.60 per cent phosphorus, and no significant difference has yet been noted in the cases of the strains or related viruses. Preparations of latent mosaic virus contained 1.1 per cent sulfur and 0.51 to 0.58 per cent phosphorus, those of tobacco ring spot 0.39 and 4.1 per cent, respectively, of sulfur and phosphorus, and those of bushy stunt virus 0.4 to 0.8 and 1.3 to 1.5 per cent, respectively, of the two elements. The chicken tumor I virus preparation contained only 0.7 per cent phosphorus, whereas the phage preparation contained 4.6 to 5.0 per cent phosphorus. All of the virus preparations were found to give a test for carbohydrate. The amount of the carbohydrate, calculated as glucose, was found to range from 1.5 per cent in the case of the phage preparation to about 16 per cent in the case of ring spot virus. All of the carbohydrate and phosphorus of tobacco mosaic and tobacco ring spot virus preparations can be accounted for by that isolated in the form of nucleic acid, hence these two viruses appear to be composed solely of nucleic and amino acids. With latent mosaic virus, only a portion of the carbohydrate appears to be in the form of nucleic acid, hence this virus must be composed of nucleoprotein plus additional carbohydrate. In the case of the phage preparation, only a small portion of the phosphorus appears to be in the form of nucleic acid, for the carbohydrate content is only about 1.5 per cent, an amount which would account for only about one-tenth of the total phosphorus. The distribution of the remaining phosphorus is unknown. The only virus preparations that have been found to contain lipoid are those of vaccinia and of chicken tumor I. It has not been determined whether the lipoid can be removed from the vaccine preparations with retention of virus activity, but in the case of the tumor agent attempts to remove the lipoid resulted in inactivation. It is possible, therefore, that lipoid may represent an integral component of the virus. MacFarlane and

Salaman (48) have reported that purified vaccine virus shows phosphatase and catalase activities but not dehydrogenase activity, and they consider that the enzymatic activities are specific properties of the virus. No other purified virus preparation has been reported to have such enzymatic activity (48a).

It is unlikely that studies on the composition of most of the viruses will consist of more than routine quantitative elementary chemical analyses in the immediate future, for at present most viruses are readily available only in amounts measurable in milligrams. However, there is no reason why the makeup of at least one typical virus, that of tobacco mosaic, should not be studied in great detail, for it is possible to obtain this virus nucleoprotein in 100-gram lots without undue effort. Preliminary studies have been completed (49), and to date the virus material has yielded only amino acids and nucleic acid on hydrolysis. The amino acids that have been identified include arginine, aspartic acid, cysteine, glutamic acid, leucine, lysine, phenylalanine, proline, serine, tryptophane, and tyrosine. Alanine, histidine, and glycine are either absent or occur in amounts that have not been measurable as yet. Doctor Ross has found the glutamic acid isolated from tobacco mosaic virus to have the customary rotation of naturally occurring d-glutamic acid (unpublished work). This is of interest because of Kögel and Erxleben's (49a) striking demonstration of the occurrence in cancerous tissues of glutamic and other amino acids having markedly lower optical rotations, indicative of the presence of the unnatural isomers. In view of the apparent dependence of insulin activity upon the presence of the disulfide linkage in the molecule, the distribution of sulfur in the tobacco mosaic virus preparation was studied (50). Of the 0.24 per cent sulfur usually found, about 0.18 per cent occurs as cysteine sulfur and 0.04 per cent or less as sulfate sulfur. Although methionine determinations frequently yield results as high as 0.04 per cent methionine sulfur the results are always somewhat lower following dialysis and it is doubtful if the virus actually contains methionine. Since virus activity is unaffected by reducing agents, disulfide groups are either absent or are not reduced or their reduction does not affect activity. It seems likely that all or most of the sulfur in tobacco mosaic virus occurs in the form of sulfhydryl groups, for the titration of the porphyrindin dye as described by Greenstein (50a) corresponds to 0.76 per cent cysteine (37).

Loring (51), in preliminary studies on the nature of tobacco mosaic virus nucleic acid, demonstrated not only that the acid is a true nucleic acid by the isolation of guanine, adenine, cytosine, and uridylic acid,

but also that the acid differs in certain respects from yeast nucleic acid. He found the diffusion constant of virus nucleic acid in 0.4 M borate buffer at pH 7.7 to be 0.10 cm.^{2}/day which, on the basis of a spherical molecule, would correspond to a molecular weight of about 37,000. The manner in which the protein and nucleic acid are combined in tobacco mosaic virus is not known. However, the fact that the two are immediately split apart on treatment with 5 per cent sodium hydroxide at 0°C., with 5 volumes of glacial acetic acid, with 36 per cent urea in 0.1 M phosphate buffer at pH 8, or on heating to 75°C., and that a fairly rapid disintegration occurs with 0.5 per cent dodecyl sulfate at pH 8 argues against the possibility that they are combined through stable chemical bonds. The fact that it has not been found possible as yet to secure evidence for the dissociation of the two in the presence of various concentrations of salt, after the manner in which sperm nucleoproteins are dissociated, indicates that the linkage is different and is probably somewhat stronger than the usual salt bonds. It is possible that the linkage is a very weak ester or amide bond or perhaps of the hydrogen bond type that is being postulated so freely at present in connection with protein structure (52). Bernal and Fankuchen (53) have deduced from x-ray data that tobacco mosaic virus contains equal sub-units about 22 x 20 x 20 A in size, and Astbury (54) on the basis of x-ray work with nucleic acids has suggested that this sub-unit, which would have a molecular weight of about 7000, must consist of one nucleotide combined with 54 amino acid residues. However, Ross has calculated from the analytical data on some of the amino acids occurring in small amounts that, if the virus is built up from similar repeat-units, the minimum molecular weight of this sub-unit must be of the order of 20 to 40 thousand. This value is in accord with Bernal's (55) recent estimate of 40,000, and with results obtained by means of the analytical ultracentrifuge on alkali dissociated virus, and by means of osmotic pressure and diffusion determinations on virus dissociated in 36 per cent urea, all of which indicated a molecular weight of the order of 50,000.

In the case of alkali degradation, evidence was obtained by means of the ultracentrifuge that the virus is first broken down into large units and these then continue to break up until eventually the small units are obtained. It has not been determined whether or not the large units still contain nucleic acid. Although it seems likely that a similar gradual breakdown occurs in the degradation of virus in concentrated urea solution, it has not been demonstrated experimentally as yet, although such studies are in progress. In no instance, other than

in Frampton and Saum's work which has not been confirmed, have the breakdown products been found to possess virus activity. Furthermore, no convincing evidence has been obtained for the reversal of the degradation process with the regaining of virus activity, although in view of the reversal of denaturation in the cases of hemoglobin and of the protein enzymes, trypsin and pepsin, it would not be unreasonable to expect that eventually conditions for the reversal in the case of viruses may be achieved. Steinhardt (56) found that the half molecules of hemoglobin obtained in 36 per cent urea still retained their characteristic absorption spectra and gas capacities and affinities. Unfortunately, it is not possible to measure virus activity in the presence of concentrated urea, but measurements made on virus degraded in urea immediately following dilution with either water or 0.1 M phosphate buffer at pH 7 showed no activity. The results indicate that the virus activity is not due to a dissociable prosthetic group, but rather to a unique architecture that is characteristic of the large molecule as a unit. Nucleic acid appears to play an important rôle in this structure, for it has been found in all of the viruses that have been purified. The nature of the combination appears to vary somewhat, for it has already been found that the nucleic acid is bound far more strongly in latent mosaic than in tobacco mosaic virus. The elucidation of the intimate structure characteristic of viruses may appear hopeless in view of the complexity and differences already found. However, if they contain somewhat similar sub-units, a study of the various degradation products should yield information concerning the general nature of the sub-units and, although the elaboration of their detailed structure may not be achieved at an early date, it is possible that the manner in which they are combined to form the virus may be learned and with this the secret of the activity.

Crystallinity and its significance. Crystalline form and the retention of constant properties following repeated crystallization have been used as criteria of purity with such great success in the chemistry of inorganic and simple organic compounds that the layman has tended to place undue confidence in them. Henderson Smith (57) has stated that there was "attached a kind of sanctity to the word 'crystal' " and that crystallinity "was a sort of a guarantee of purity." However, chemists, and especially protein chemists, who are familiar with the great tendency of proteins to form solid solutions and to carry along impurities on crystallization, have long recognized that, while crystallinity and the retention of constant properties on repeated crystallization are very useful tests in the determination of the purity of a material, they are

not infallible tests, they are less well suited for purity tests in the case of proteins, and most certainly they are not an absolute guarantee of purity. Nevertheless, following the announcement in 1935 of the "isolation of a crystalline protein possessing the properties of tobacco mosaic virus," the fact that the material could be obtained in crystalline form was more instrumental than any other in securing the acceptance of the material as pure and as the virus, although to the writer and to many others it was perhaps the least convincing of the experimental evidence. The fact that the protein could be repeatedly crystallized with retention of constant properties was a more significant bit of evidence, but even in this first paper the possibility of a solid solution of different active and inactive proteins was mentioned. The crystallinity of the protein also caused many individuals to decide that the virus was non-living because they considered it impossible for living organisms to take on a crystalline structure. However, it has been shown that crystallinity is simply a structural regularity and that actually there need be no incompatibility between the living and the crystalline states (58). Nevertheless, if the protein had not been obtained in crystalline form it is very probable that, because of the general conception of viruses as living organisms, it would have been generally considered as a minute living organism. The crystallinity focussed attention on the other physical and chemical properties of the protein and, although some workers doubted that the virus activity was a property of the protein, the material was generally accepted as being composed essentially of large protein molecules. Because all of the evidence indicated that the virus activity was a specific property of the large protein molecules, and because, at that time, the choice appeared to be only between a living and a non-living agent, the writer joined in the acceptance of the virus as a non-living protein. However, a growing realization of the futility of attempting to distinguish between the properties of non-living and of living things and an increased store of knowledge of viruses have permitted a newer and more exact definition of the general nature of viruses, which will be considered in a subsequent paragraph. Here it may be concluded that crystallinity, of itself, offers no evidence as to the living or non-living nature of a material and is no absolute guarantee of purity, and that retention of constant properties following repeated crystallization should be regarded as merely one of a great many tests used to determine purity, any one of which may be fallible and all of which should be used before a decision regarding purity is reached.

It has not been found possible to crystallize some of the unstable

virus proteins, and it is likely that more viruses will be isolated which can not be crystallized. In the cases of unstable viruses, the continual breakdown may provide sufficient impurity to prevent crystallization, and in other cases it is possible that pure preparations may be obtained which will fail to crystallize, hence failure to obtain a virus in crystalline form should not be considered as a definite indication of an impurity. In his excellent review on the nature of viruses, Levaditi (59) points out that no virus affecting animals or man has been obtained in crystalline form and considers that the crystallinity of tobacco mosaic virus and the lack of crystallinity in the case of the papilloma virus preparations represent an important difference. It may be noted that the same difference exists in the case of tobacco mosaic and tobacco ring spot viruses, very probably because of the instability of the latter virus. If a virus preparation fails to crystallize but is found to be homogeneous by all other tests, it should be regarded as a pure material. Should the papilloma virus be found to have chemical properties similar to those of tobacco mosaic virus and to be homogeneous by all available tests, the mere lack of crystallinity would not be a valid reason for differentiating between it and tobacco mosaic virus. There is good reason, therefore, to subject the purified preparations of vaccine, Shope rabbit papilloma, chicken tumor I, and foot-and-mouth disease viruses to the various tests for homogeneity, for in every case it should be possible to determine whether or not the preparations are pure and hence represent the virus. So far, there is no good reason to attempt to differentiate viruses affecting plants from those affecting man and animals.

Tobacco mosaic and its strains, aucuba, masked, and enation mosaic viruses, the closely related cucumber mosaic 3 and 4 viruses, and bushy stunt of tomato virus and tobacco necrosis virus have all been obtained in the form of crystals readily visible under the microscope. All of the first named viruses were obtained in the form of long thin pointed needles, while the bushy stunt virus was obtained in the form of rhombic dodecahedra and the tobacco necrosis virus as thin plates. Latent mosaic of potato virus has not been obtained in the form of distinct crystals, but the pellet obtained on high speed centrifugation was found to be doubly refracting. When 1 to 2 per cent solutions of latent mosaic virus or of tobacco mosaic virus or its strains are allowed to stand, they gradually separate out into two distinct layers, the lower of which is liquid crystalline. No purified viruses other than those mentioned above have been obtained in crystalline form to date. Bernal and Fankuchen (53) made x-ray diffraction studies on oriented crystals and

solutions of virus preparations and found no differences between strains of latent mosaic virus and only doubtful ones in the case of the cucumber mosaic 3 and 4 viruses. However, they found definite differences in intensity of the intermolecular pattern in the case of the strains of tobacco mosaic virus, although the intramolecular pattern appeared to be the same. In every case the intramolecular pattern was found to be independent of the concentration of the virus while the intermolecular pattern, on the other hand, varied continuously and quantitatively with the concentration. The intramolecular pattern of the bushy stunt virus was found to be of essentially the same type as that of tobacco mosaic virus, indicative of a repeat-unit of approximately 20 x 20 x 22 Å. Bernal (55) came to the interesting conclusion that the individual molecules of tobacco mosaic virus have an internal crystalline structure, that this structure is analogous to that of other crystalline proteins, and hence that each molecule may be regarded as a crystal. Although the beautiful dodecahedric crystals of bushy stunt virus are regarded as true crystals, Bernal considers the intermolecular pattern of crystalline tobacco mosaic virus to indicate the presence of long molecules arranged with a perfect hexagonal, 2-dimensional regularity at right angles to the length but with no regularity in the direction of the length. He is of the opinion that the solid needle-shaped particles visible under the microscope which have been referred to as crystals have only this type of regularity and are therefore really in a liquid crystalline state and should be referred to as liquid or para-crystals. Wyckoff and Corey (60) have also studied the x-ray diffraction pattern of crystalline tobacco mosaic virus and, although they obtained essentially the same pattern as that reported later by Bernal, they interpreted it as resulting from true crystals.

Recently, Kausche (61) reported that he had succeeded in preparing *in vitro* the hexagonal crystals of tobacco mosaic virus, which previously had been noted only within living cells (62). Although proof of identity was not offered in the preliminary paper, it seems likely that the crystals are composed of virus and that the necessary proof will be forthcoming soon. Beale (63) had previously described the transformation of the hexagonal crystals within living cells into the needle crystals upon the addition of acid. Kausche reported that he had observed under the microscope the reversal of this phenomenon, namely, that bundles of the needle crystals fused together to form the hexagonal crystals. He considers that the molecules, the short and long fibres or filaments described by Best (64), the needle crystals, and the hexagonal

crystals form an unbroken series. The preparation of the hexagonal crystals *in vitro* should make it possible to determine whether or not they are more complex in composition than the needle crystals, a possibility which was suggested by Beale. Bernal (65) has stated that the hexagonal crystals occurring within cells "possess end as well as side faces and undeniably show 3-dimensional regularity" and Bawden (66) also considers them to be true crystals. However, Bernal and Bawden consider that the needle crystals are not true crystals and possess only a 2-dimensional regularity, hence, if the hexagonal crystals are formed from the needle crystals, a rather unusual realignment of the molecules must occur. It appears preferable to leave the question of the kind of crystallinity open for the present. Whether crystalline tobacco mosaic virus possesses a 2- or a 3-dimensional type of regularity is not important from the standpoint of the virus worker, even though it may be quite important to the crystallographer for, as mentioned above, crystallinity is not a vital issue with respect to either the purity or the nature of viruses, hence regardless of the final decision the general virus problem will remain unchanged.

Size and shape. Most of our knowledge concerning the sizes of viruses has been obtained by Elford (67) using the method of ultrafiltration analysis. However, supplementary information has been obtained by means of ultraviolet light photography, and since the isolation of purified viruses in weighable amounts the excellent method of ultracentrifugal analysis of Svedberg (68) has been used with great success and will undoubtedly be used more and more in the study of viruses. It has been found that viruses may be arranged in an unbroken series with respect to size from those, such as psittacosis virus, that are about 275 mμ in diameter, down to those, such as poliomyelitis and foot-and-mouth disease viruses, that are only about 10 mμ in diameter. It should be noted that some viruses are larger than accepted living organisms, such as agalactia of sheep, bovine pleuropneumonia, and sewage organisms, which are about 175 mμ in diameter, while other viruses are smaller than accepted protein molecules, such as those of the hemocyanin proteins. Viruses, therefore, form an unbroken series from accepted organisms to accepted protein molecules, and there is an overlapping at either end. A given virus appears to have a definite and characteristic size regardless of its source, and the strains of a given virus appear to have essentially the same size. Another point which should be noted is that viruses exhibiting similar clinical behaviors, such as vesicular stomatitis and foot-and-mouth disease viruses, may

nevertheless vary widely in size. The measurements of the sizes of viruses by ultrafiltration, which constituted the earliest reasonably exact physical measurements on viruses, and the demonstration that some viruses were smaller than protein molecules and that viruses differed in mass by a factor of about 10,000 represented most important advances.

In most of the work on the estimation of the sizes of viruses, it has been tacitly assumed that the virus particles were essentially spherical in shape. Recently, however, it was demonstrated that some viruses are very asymmetrical in shape (69). The earliest indication of the asymmetry of a virus was obtained by Takahashi and Rawlins (70), who noted that double refraction of flow was exhibited by the juice from a plant diseased with tobacco mosaic virus but not by the juice from normal plants. In view of Freundlich's (71) work on the vanadium pentoxide sols and because of the nature of the double refraction of flow, they concluded that tobacco mosaic virus or some material regularly associated with it was composed of rod-shaped particles. Since that time and following the isolation of the virus in purified form, an imposing mass of evidence of a more or less indirect nature has been obtained, chiefly by Lauffer (72), that tobacco mosaic virus consists of molecules having a cross section of about 12 mμ and a length of the order of 400 mμ. The strains of tobacco mosaic virus, the related cucumber mosaic 3 and 4 viruses, severe etch virus, and latent mosaic of potato virus have also been found to have similar rod-like shapes. Direct and striking confirmation of this general conclusion was recently achieved when, by means of the electron microscope, Kausche, Pfankuch, and Ruska (72a) found the probable cross section and length of tobacco mosaic virus to be about 15 mμ and 300 mμ, respectively. Tobacco ring spot, rabbit papilloma, vaccinia, and bushy stunt of tomato viruses have not been found to show double refraction of flow and hence probably have relatively symmetrical shapes. The marked asymmetry of some of the viruses has thrown doubt upon sizes estimated by ultrafiltration or by ultracentrifugation, since few studies have been made on the manner in which rod-shaped particles filter or sediment. However, in the case of tobacco mosaic virus, Lauffer (73) has shown that different methods of estimating molecular weight from viscosity, sedimentation and diffusion data, one of which is independent of the shape of the molecules, give values that agree closely. The cross section of the molecule, calculated from the length to width ratio of 35:1 obtained from viscosity studies and the molecular weight of about 50×10^6,

is 12.3 mμ, which is in agreement with the results of ultrafiltration, if it be assumed that the width of the particle represents the limiting dimension. This value for the cross section is approximately the same as that of 15.2 mμ suggested by Bernal on the basis of x-ray data and that of 12.5 mμ suggested by Langmuir and Schaefer (74) as a result of studies on monolayers of the virus. Smith and MacClement (75) have reported that tobacco mosaic virus can be drawn through a membrane having an average pore size of only 13 mμ by means of an electrical field. Nothing is known about the relationship of particle size to pore size under such conditions, but, if it be assumed that such pores represent the minimum cross section of the virus, a size in agreement with that mentioned above is obtained. It has been demonstrated, therefore, that viruses vary in shape and in size from psittacosis and vaccine viruses with essentially spherical shapes and diameters of about 275 and 175 mμ, respectively, through a gradual series of viruses of fairly well established sizes but of unknown shapes; to tobacco mosaic virus with a molecular weight of about 50×10^6, a width of about 12 mμ, and a length of about 400 mμ; on through a group of small spherical viruses such as those of the rabbit papilloma, tomato bushy stunt, and tobacco ring spot, having diameters of 40, 28 and 19 mμ, respectively; down to the very small poliomyelitis and foot-and-mouth disease viruses having molecular weights of 700,000 and 400,000, respectively, and effective diameters of 12 and 10 mμ, respectively, but with the actual shapes unknown.

An interesting phenomenon shown by viruses having a very asymmetric shape and apparently directly dependent upon a rod-like shape, is the formation of two distinct layers when rather concentrated solutions of such viruses are allowed to stand (5). The line of demarcation is very sharp and gradually rises with time. The upper layer is the more dilute and shows double refraction only when caused to flow, whereas the lower layer is the more concentrated and is spontaneously doubly refracting. The lower layer appears to result when the rod-shaped virus molecules become sufficiently concentrated so that they lose their ability to rotate about their two shorter axes, and appears to consist, therefore, of a 3-dimensional mosaic of regions arranged at random to each other but in each of which all of the molecules lie approximately parallel. The phenomenon appears to be quite analogous to that first reported by Zocher and Jacobsohn (76) for vanadium pentoxide sols. Lauffer (77) concluded that the lower layer represents a special case of double refraction because it has no extinction direction.

He found the double refraction exhibited by tobacco mosaic virus to be due largely, if not entirely, to the shape of the particles and scarcely, if at all, to intrinsic double refraction. The layering phenomenon as well as the variation of the intermolecular x-ray pattern with concentration of virus is also of interest in connection with the long range electrical forces which have been the subject of several recent theoretical papers (77a, 77b). It has been suggested that these electrical forces could be effective over unusually long distances and the results with tobacco mosaic virus provide experimental evidence for the existence of forces effective between molecules over distances of a hundred or more Å. The reproduction of viruses as well as the duplication of cells may be dependent upon such forces.

Bawden and Pirie (5) found that the purification of tobacco mosaic virus by chemical means resulted in a loss in activity and filterability and an increase in stream double refraction and concluded that the purification had caused an aggregation of the virus. Similar results have been reported in the cases of other viruses, and as a result there has been a tendency on the part of virus workers to assume that purification as such must invariably cause aggregation of virus and hence that it is impossible to isolate virus in an unaltered condition. The question of the aggregation of tobacco mosaic virus was studied by the writer with Loring and Lauffer (26), and it was found that virus purified by rapid chemical treatment in the cold, or preferably by means of ultracentrifugation in the cold, was entirely comparable to the virus in untreated juice with respect to specific activity, filterability, and stream double refraction. However, merely allowing such purified virus to stand for a short time in the presence of low concentrations of salt at room temperature was sufficient to cause aggregation. Similar results have been obtained with latent mosaic and tobacco ring spot viruses. It should be recognized, therefore, that, although viruses may be aggregated readily by chemical treatment and most of the chemically purified virus preparations that have been isolated have consisted of aggregated virus, it is possible nevertheless to isolate tobacco mosaic virus in a state and possessing a specific activity, filterability, and stream double refraction comparable to that of virus in unpurified preparations.

The occasional suggestions that, in untreated juice, tobacco mosaic virus consists of small units or that active low molecular weight material may be produced following a given treatment are not in accord with results obtained in the writer's laboratory. If active low molecular weight material existed, it would not be possible to sediment the active

units in a centrifuge at a speed at which other low molecular weight material, such as hemoglobin, fails to sediment. However, it is an experimental fact that, when untreated juice or preparations supposed to contain low molecular weight active material are mixed with hemoglobin and centrifuged at a speed which will sediment ordinary tobacco mosaic virus but not hemoglobin, the virus activity is found in the sediment and not with the unsedimented hemoglobin in the supernatant liquid. This is good evidence that virus in untreated juice does not consist of low molecular weight material. Similar results have been obtained by Hughes, Pickels, and Horsfall (78) in a study on the differential centrifugation of proteins. These workers demonstrated that hemocyanin could be separated from egg albumin and that yellow fever virus could be separated from serum protein without any difficulty by means of differential centrifugation. It may be concluded that to date there has been no convincing demonstration of the existence or production of low molecular weight material carrying virus activity.

Nature and mode of action of viruses. The isolation of several of the viruses in the form of high molecular weight proteins and the demonstration, beyond a reasonable doubt, that the preparations are essentially pure and that virus activity is a specific property of the proteins necessitate a consideration of virus activity in terms of these materials. The viruses vary in composition from the amino and nucleic acids of tobacco mosaic and tobacco ring spot viruses to the amino acids, nucleic acid, carbohydrate, and lipoid that go to make up vaccine virus. Despite the variation in composition and size, there is no reason to believe that viruses differ among themselves in any fundamental respect, or that there is other than a continuity of structure from small to large viruses. All viruses have in common the ability to reproduce or multiply when placed within susceptible living cells and, since no virus has been found to multiply under any other conditions, they may be considered obligate parasites. The manner in which viruses multiply has been, and remains, a matter of much conjecture. In the case of phage, Northrop (12) considers that multiplication may be more simply explained by analogy with the autocatalytic formation of pepsin and trypsin than by analogy with the far more complicated system of living organisms. This seems a reasonable attitude, especially in view of Krueger's (79, 80) evidence for the existence of an inactive precursor or pro-phage. However, many difficulties become apparent when this viewpoint is adopted for viruses in general, for no evidence has been obtained for the existence of inactive precursors having chemical properties similar

to those of the viruses, no virus has been produced *de novo* or in the absence of living cells, and a multiplicity of precursors must be postulated, since a given host cell is capable of producing any one of hundreds of different viruses or virus strains.

Nevertheless, it is quite possible that the basic idea of catalysis may be correct with respect to virus activity. Bergmann (81) has shown experimentally that a catalyst, a protein enzyme, can cause the formation or synthesis of a peptide linkage. There is every reason to believe that proteins are produced through the formation of peptide linkages, hence it is but a step to consider that proteins may result from the catalytic action of still other proteins, and but another step to consider that a protein may catalyze reactions resulting in the formation of replicas of itself. The latter protein, catalyzing such reactions within a cell, would conform, of course, to our definition of a virus. The virus reaction may, therefore, resemble the pepsin and trypsin activation reactions and the reaction postulated for phage, except that the virus reaction is far more complicated, requiring not one but a series of reactions and special conditions which so far have not been reproduced outside of a cell. However, the great advances that have been made in recent years with respect to our knowledge of enzymes, hormones, organizers, and genes, and the work of Keilin (82), Warburg (83), Green (84), and others on intracellular reactions lead one to suspect that elucidation of the virus reaction is not beyond our grasp.

The introduction of a virus most certainly diverts the normal metabolic activity of a cell, yet the influence of the virus might be likened to that of agents already present which direct normal metabolism except that the virus exerts a dominating influence. The mechanism by means of which virus is synthesized within a diseased cell must be very similar to that by means of which normal proteins and constituents are synthesized within a normal cell. Levaditi (59) has postulated the existence of two factors within cells, a "constructive" and a "lytic" factor, and supposes that in the case of a virus-infected cell the "constructive" factor of the virus superimposes itself and dominates the normal factor. Similar ideas have been advanced in the writings of other workers, especially when cancerous cells were under consideration (85–87). In some instances the loss, rather than the addition, of a factor has been postulated. One great point of difference is that in the case of the virus reaction the "key" to the disrupted metabolism, that is to say, the virus, can be separated, isolated in pure form, and studied apart from the system, whereas in the cases of normal cells or of cancerous cells

similar "keys" have not been found separable as yet. The "key or keys" must be contained within the chromosome and probably are represented by genes or gene derivatives, hence they may be nucleoproteins. The fact that all viruses so far isolated have been found to consist of or to contain nucleoprotein may be of special significance, and the possibility that they may have been derived from genes or nuclear material has been considered from time to time by different writers (88–92). Although it is quite possible that viruses may have arisen originally in such an endogenous manner, it should be emphasized that, as recognized today, virus activity results only from the introduction of virus from without. There is, however, a strong and growing tendency to consider that viruses or similar factors may, upon provocation of the cell, originate endogenously and give rise to tumors or cancers (93–95). Whether such viruses are actually derived from normal cell constituents or are formed by the mutation of a "masked" virus normally or usually carried within the cell, but in reality alien to the cell, is not known. The probability that the synthesis of viruses does not differ fundamentally from the synthesis of normal proteins within cells and the fact that, although viruses may be removed and studied apart, they nevertheless possess most of the properties of living organisms, cause the attack on the nature and mode of action of viruses to become of signal importance, for it is in reality an attack not only on the problems of abnormal and normal metabolism, but also on the nature of life. The difficulties, discussed by Bohr (96), involved in making studies of the living state without affecting conditions by the very act of study, are lessened in the case of viruses, since they may be removed from cells, subjected to various studies, and then reintroduced into cells without measurably affecting them. This property of suspended animation is unique with viruses and is possessed by no other entity to a similar degree, for the same difference that exists between viruses and ordinary living organisms must exist between viruses and the seeds or spores that are usually cited as examples of suspended animation. This and other preceding statements are necessarily limited by one's understanding of the nature of the difference between viruses and living organisms, hence this question will be considered.

The chemist, after a perusal of the properties of the purified viruses that have been isolated and adequately studied, has no difficulty in coming to the conclusion that they are protein molecules. The chemical and physical properties are admittedly those to be expected of large protein molecules, and the chemist, well acquainted with the catalytic

synthesis of a peptide bond and the autocatalytic formation *in vitro* of the pepsin and trypsin proteins, views virus activity as but a logical and not unexpected extension of the expression of chemical structure. True, the duplication of conditions necessary for the expression of virus activity *in vitro* has not yet been accomplished and but little has been done with the large and complex viruses such as vaccine virus, but the chemist, secure in the knowledge already gained, considers that such problems lend themselves to experimentation and looks forward with confidence to their eventual solution.

The pathologist and biologist, long interested in the expression of virus activity and its results, have, on the other hand, considered viruses to be small living organisms. Recently, however, there has been a tendency on the part of some workers to accept the view that some or all viruses may be something other than living organisms. Rivers (97) has suggested that the large viruses may be small living organisms, the middle-sized viruses representatives of an unknown form of life, and the small viruses non-living agents, but that it is impossible to draw lines dividing the groups and that very probably one group shades off into its neighbors. Rivers appears to believe, therefore, that, although the large differ from the small viruses, they nevertheless form a continuum from small non-living to large living viruses. Green (98) and others have suggested that viruses are simplified fragments of living protoplasm arising from organisms by a process of retrograde evolution under parasitism involving loss of function and associated substance, and that this process may result in forms varying from a single colloidal molecule to entities almost indistinguishable from ordinary living organisms. Laidlaw (99) has presented a similar view and has assembled much evidence in its support. Although he rejects the simple "autocatalytic protein" view of viruses, he supposes that viruses arise by a gradual loss of substance and of such functions as enzyme systems. He offers no alternative theory of the mechanism of virus action and appears to consider that viruses are formed much as normal cellular constituents are formed. As mentioned above, this view, because of the recent work on intracellular reactions, must have catalysis as its basis. The general situation with respect to bacteriophage has been quite comparable, for d'Hérelle (100) first postulated that phages were sub-microscopic living entities, probably constituted of simple colloidal micellae, and later Bordet (90) considered them to be non-living transmissible lytic principles. Wollman (91) regarded phages as being derived from the bacteria and discussed the possibility of their being

similar in nature to a gene. Doerr (101) has just published a masterly discussion of the nature of viruses and bacteriophages in which he has presented and evaluated the ideas held by different workers. He points out that the large viruses are similar to the small viruses in all important respects, that there is no reason for attempting to separate them on the basis of size, and that, furthermore, no fundamental difference between viruses and living organisms has been demonstrated. In Doerr's opinion, the chief point of issue is whether non-living infectious agents should be accepted along with living infectious entities or the smallest virus accepted as a living agent, and after a thorough discussion he tends to resolve the conflict in favor of the acceptance of viruses, even though they may consist of a protein molecule, as living. The pathologist has been able to rationalize his knowledge of viruses with his knowledge of living organisms so that he is willing to accept the smallest virus, even though a large nucleoprotein molecule, and the largest living organism as representatives of a common series in which there is a definite but almost imperceptible gradation of substance and of complexity of function.

We come, therefore, to the interesting situation where the pathologist and the chemist have a common meeting ground, a territory never before accessible with certainty. Needham (102) has argued the necessity of a bridge between morphology and biochemistry, and the essence of this bridge now appears to have been achieved between pathology and chemistry. The chemist, always interested in atoms and molecules, has recently extended his knowledge in two directions. At one extreme, nuclear chemistry has achieved the transmutation of elements and at the present time is pushing vigorously ahead with studies on the manner in which the protons and electrons that make up the atoms are arranged and on ways and means of breaking up large and complex structures into simpler ones. Instead of being homogeneous, the atoms appear to consist of a virtual maze of discontinuities, but nevertheless in perfect order as shown by the breakdown of the large uranium atom into a barium atom. At the other extreme, the chemist has found the virus proteins, molecules larger than those ever known before. However, the chemist realizes that just as the chemical, biological, and physical properties of ordinary molecules are a direct result of their structure, so too must the properties of atoms and of the viruses be a direct result of their structure. This structure, whether evidenced by the proton and electron of the hydrogen atom, by the atoms of the water molecule, or by the units combined to make up a

virus, must be fundamentally the same. The recognition of the essential identity of the structure of entities, regardless of their nature, that is, of structure as a continuum from smallest to largest, is of fundamental importance. Although recognizing the significance and importance of structure, many have been led astray because of attempts to separate the living from the non-living on the basis of certain characteristics, not realizing that, as Pirie has so well stated in his essay on the subject, the word "life" is merely a definition of degree. Aristotle is credited with saying, over two thousand years ago, that "Nature makes so gradual a transition from the inanimate to the animate kingdom that the boundary lines which separate them are indistinct and doubtful." Attempts to arrive at a definite line of division have resulted in failure in the past and appear doomed to a similar fate in the future. Fortunately, the classification of an entity as living or non-living, or as a cell or a molecule, is of little or no importance, whereas the complete realization of the expression inherent in structure is of tremendous importance.

It has been stated before (103) that from the standpoint of structure there is no reason why a single structural entity, which we call a molecule, should not be larger than the ordered group of structural entities which we call a cell. The overlapping of structure in the case of atoms is well known, and the recent demonstration of the formation of barium and other atoms from a single uranium atom brings it even more forcibly to attention. A similar situation may prevail at the other extreme of size. Neither the cell nor the atomic theory should be handicapped by a reference to the living state, but should be utilized only to define certain accepted orders of structure. It should also be recognized that there may be such a gradual transition between these accepted orders of structure that the designation of intervening entities as molecules or as cells becomes one merely of personal preference. The nature of the bonds between units within cells, as well as the nature of the bonds which hold together the large nucleoproteins, requires much more investigation before they may be completely understood. However, at the present time there is no reason to believe that they differ in any fundamental respect from the forces already known to exist in atoms. It becomes possible, therefore, to blend the atomic theory, the germ theory, and the cell theory into a unified philosophy, the essence of which is structure or architecture. The chemical, biological, and physical properties of matter, whether atoms, molecules, germs, or cells, are directly dependent upon the structure of the matter, and the results of

the work with viruses have permitted the conclusion that this structure is fundamentally the same regardless of its occurrence.

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FAT TRANSPORT IN THE ANIMAL BODY

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The problem of fat transport differs essentially from that of protein or carbohydrate transport in that the fatty acids, the elemental constituents of the fat, are insoluble in water as are also most of their compounds, while the corresponding elements of protein and carbohydrate are readily water-soluble. The digestion products of the proteins and carbohydrates enter the blood directly and are carried as such, while the fats are resynthesized during absorption, are transported in the lymph, and are discharged into the blood and distributed mainly as fat in a fine state of division. The mechanisms provided in the animal body for the carriage of the fatty acids and their compounds in the blood and across the various cellular barriers are consequently quite different from those provided for the other foodstuffs. Conceptions of the method of transport have changed considerably in the fifteen years since the last review was written. Important new tools for use in the study of fat transport have been found in the tagged or labelled fatty acids which it is now possible to trace on their way through the organism, and in radioactive phosphorus, used first by Hevesy (1935) and later by many workers including Perlman and Chaikoff (1939a), Artom, Sarzana and Segré (1938), Chargaff (1939) and Haven and Bale (1939), which combines with the fatty acids in the lecithin type of combination. The marked fatty acids used are: 1, the iodized acids as used by Artom (1933a); 2, highly unsaturated acids as used by Sinclair (1929); 3, elaidic acid by Sinclair (1935b) and Kohl (1938); 4, acids containing deuterium (Schoenheimer and Rittenberg, 1935; Barrett, Best and Ridout, 1938; Cavanagh and Raper, 1939); and 5, fatty acids with characteristic absorption spectra such as eleostearic acid and activated linoleic acid by Burr and associates (Miller, Barnes, Kass and Burr, 1939). It is proposed in the present review to sketch briefly the new facts and ideas in their bearing on the problem.

TRANSFER ACROSS THE INTESTINAL MUCOUS MEMBRANE—FAT ABSORPTION. It is well recognized that the bile and the lipases of the

pancreas and intestine are important parts of the mechanism for the transfer of fat from the intestine to the blood. Work in the last few years has provided a fresh insight into the mechanism of their activity. In addition, the phosphorylation of the fats—the formation of the more mobile and perhaps more reactive phospholipids—has introduced a new factor which is perhaps more important than the known ones with respect to fat transport.

Fat splitting. As was concluded in the previous review of this topic, practically all evidence is in favor of a complete splitting of the fat during its stay in the normal intestine. Even in those cases in which there is little absorption, it is the exception to find unsplit fat in the feces. For that reason it can be assumed that the first stage in the transport of fat from the intestine is complete splitting into its constituent fatty acids and glycerol. The same holds for other hydrolyzable fatty acid compounds, the phospholipids, lecithin and cephalin. The difficultly hydrolyzable fatty acid compounds, as far as they have been investigated, such as sphingomyelin, cerebrosides, and cholesterol esters, are not absorbed.

Soap formation. Much has been made of the fact that the intestine is slightly acid (pH 6.7) throughout most of its length, from which it was concluded that there could be little if any soaps present and there could then be little absorption of the fatty acids in the form of the water-soluble soaps. On the other hand, the compounds of the fatty acids with bile salts are stable and diffusible down to a pH of 6.2 and these compounds rather than the soaps are now regarded as the form of absorption of the fatty acids. Soaps are probably essential in the primary emulsification of fat required for its rapid hydrolysis by the lipase. The lipases being water-soluble have contact with the fat only at its surface. Hence, it is desirable that the surface be made as great as possible, which is accomplished by the emulsification. After the lipase has made contact with the fine fat particles, it is probable that splitting is rapid and a continued state of emulsification is not necessary. Since both the pancreatic juice and bile are alkaline at the time of entry into the intestine, conditions at that time are right for soap formation by combination of the secreted alkali with the free fatty acid always present in the food fat. Emulsification of the unsplit fat could then take place and probably persist long enough for hydrolysis to take place.

The train of events in the absorption of fat from the intestine may then be outlined as follows. The fat of the food, which in ordinary diets does not constitute more than 15 per cent of the dry weight of the

food, always contains a small percentage (less than 10 per cent) of free fatty acid. This amount is increased to a variable extent during its stay in the stomach, due in part to the gastric lipase. Fat in the food slows the emptying of the stomach so that the fat is delivered to the intestine in small portions. In the duodenum, it meets the alkaline secretions of the pancreas and liver, and the free fatty acid is neutralized forming soap which rapidly emulsifies the unsplit fat. At the same time the emulsified fat is mixed with the pancreatic lipase and digestion proceeds rapidly. The fatty acids and soaps formed by the digestion combine with the bile salts and are absorbed with their help. The removal of the split-products by absorption speeds up the hydrolysis of the unsplit fat. Making use of the labelled fat elaidin, Kohl (1938) showed that, after an initial lag, fat is absorbed from the intestine of rats at a nearly constant rate.

Whether any unsplit fat is absorbed as such, swept in with the current of split-products or by direct penetration, cannot be stated, but from present evidence as regards normal intestinal behavior it seems unlikely. Evidence of direct absorption of fat without hydrolysis under certain conditions continues to appear. Kitagawa (1934) reported that finely dispersed fat was directly absorbed without preliminary hydrolysis from the large intestine and the lower part of the small intestine of dogs. Onozaki (1936) obtained similar results. The absorption of even the very finely divided fat was very slow—from $\frac{1}{8}$ to $\frac{1}{16}$ as fast as absorption after splitting. Doubilet and Reiner (1937), taking advantage of an accidental Thiry-Vella fistula in the middle ileum in a human being, found that oleic acid and olive oil were readily absorbed in the absence of bile acids and that there was but little improvement when bile acids were used. On the other hand, Riegel, Elsom and Ravdin (1935) found that while the absorption of oleic acid from jejunal loops in dogs was slight, the presence of sodium taurocholate and to a less extent hepatic or gall bladder bile greatly increased the absorption.

Different parts of the intestine probably behave differently as regards the absorption of unsplit fat. Also the possibility of splitting by lipases in the intestinal juice has not been excluded.

Bile. The importance of bile in fat digestion and absorption has been recognized for a long time and it is probably the greatest single factor in the transport of fat from the intestinal lumen into the absorbing cells. It is more important than the pancreatic lipase since the functions of this secretion can be taken over by other lipases in the intestine.

Work which throws light on the part taken by the bile was supplied

by Verzár and Kúthy (1929) who found that sodium salts of glycocholic and taurocholic acids dissolve palmitic and stearic acids and that the combination is diffusible and therefore molecularly dispersed. The solution was found to be stable in buffers at pH 6.2, i.e., at an acidity greater than prevails in the small intestine. These findings offer an explanation of the effect of bile on fat absorption and also dispose of the difficulty raised by the acidity of the intestinal contents which would make difficult or impossible the preservation of a soap emulsion in the intestine. Verzár and Kúthy thought at first that the association products of bile salts and fatty acids, i.e., compounds containing as much as 8 molecules of bile salt to 1 of fatty acid were absorbed as such. Calculation showed them that this assumption would require an amount of bile salt much greater than could be supplied by the normal secretion of bile and would require either a circulation of bile salts or some other mechanism of absorption. The hypothesis finally offered was that the bile salts become fixed in the intestinal mucous membrane and by their solvent effect bring about the passage of the fatty acids into the epithelial cells. The finding of Breusch (1937) that saturated fatty acids from C_{16} upwards and also oleic acid in presence of bile salts were not diffusible through parchment was explained by Verzár as due to the low permeability of Breusch's membranes. Breusch found further that the saturated acids below C_{16} down to C_{10} were increasingly diffusible while the solubility in bile salts decreased with increasing molecular weight. Of the acids of lower molecular weight, the soaps were often much more diffusible than the bile salt. These experiments were made with parchment membranes and the behavior of such membranes may be quite different from that of the living intestinal epithelium. Other authors (Szörényi, 1932; Müller, 1932) confirm Verzár and Kúthy in finding that the higher fatty acid compounds are diffusible. Whether or not lipid material can be moved across membranes seems to depend on its chemical nature, probably on its ability to combine with substances in the membranes which act as carriers, e.g., protein, bile salts, etc. Breusch found a quite sharp specificity in absorption of material from the fat stores. Fat and cholesterol moved readily while paraffin did not. Cholesterol was absorbed from the intestine while coprosterol was not.

Absorption into the portal circulation. Water-soluble metabolites absorbed from the intestine are collected by the portal system and carried to the liver. Relatively little is absorbed by the lymph system. The water-insoluble fats, by contrast, are largely absorbed via the lymph system and reach the blood indirectly. It is still a disputed

point whether any appreciable amount of fat is absorbed directly into the blood. The observation which, more than any other, keeps alive the dispute is the old one of Munk and Rosenstein who were able to recover only 60 per cent of the absorbed fat by a chyle fistula. It was not certain in their subject that the fistula delivered all the chyle; some may have passed into the blood by other channels, so that there is a good possibility that there may not have been any appreciable direct absorption. Nevertheless, the missing 40 per cent has stimulated efforts to account for it mainly by demonstrating an increase in the fat of the portal system over that in the general circulation, which would indicate a direct absorption into the blood. Water-soluble compounds of the fatty acids, such as the soaps of the short-chain fatty acids or of such oxidized fatty acids as are believed by Tangl and Berend (1930) to be formed during fat digestion would be expected to appear in the portal system, but in the digestion of ordinary fat it has not been demonstrated that such compounds are formed.

Brockett, Spiers and Himwich (1934) found no change in the lipid content of serum of portal blood during absorption of oil, which is in agreement with the results of most preceding work. On the other hand, Cantoni (1928) found that the portal blood contained definitely more fatty acid and less cholesterol than the carotid blood. The absorption of fat directly into the blood in the form of phospholipid is a possibility which has not yet been investigated. Eckstein (1925), after diverting the lymph of the thoracic duct by fistula, found a small but appreciable increase in the total fatty acids of the blood during fat absorption while there was no change in the blood of the controls not fed fat. The situation as regards absorption of fat directly into the blood remains unsettled.

Histological investigations of fat absorption. Rossi (1936), confirming his earlier work, found that the intestinal mucosa on immersion in a solution of fatty acids in bile salts, even when fixed with formaldehyde, could take up the fatty acids which were demonstrable as fine granules inside the cells. Neutral fat dissolved in bile acids did not diffuse. Free fatty acids alone diffused but much more slowly. Treatment of the formalin-fixed intestine with fat solvents destroyed its power to take up the fatty acids, which led to the conclusion that the lipid of the absorbing cells was the effective agent in the absorption. Jeker (1936) and Verzár and Jeker (1936) reported experiments which appeared to show the rapid absorption of fatty acid from the intestine and its later change into fat. For the demonstration of fatty acids, Fischler's stain

was used but in view of the criticism of Fischler's stain by Kaufmann and Lehmann (1926) which Jeker and Verzár did not attempt to meet, their conclusions are doubtful. On the other hand, the photographs of the sections make it certain that some material, presumably fatty acid, appears in the epithelial cells within a few minutes after exposure to the fatty acid-bile mixture and that the absorbed material undergoes a change which makes it no longer stainable with Fischler's stain but stainable with Sudan. The change was prevented by iodoacetate and by adrenalectomy and restored by injections of adrenal cortical hormone.

THE LIVER AND FAT TRANSPORT. That the liver takes an active part in fat transport is generally accepted. The old observation of a mobilization of fat to the liver in starvation and under the action of various drugs was interpreted as a step in the preparation of fat for metabolic use. The liver also acts as a temporary storage place for fat when it is being absorbed from the intestine just as it acts as a temporary store for carbohydrate and protein products. The nature of the changes brought about in the fat by the liver during the period of storage is becoming apparent from recent work and appears to be essentially phosphorylation, a change into lecithin, which was the assumption made on purely theoretical grounds by the earlier workers. Moreover, it is becoming increasingly apparent that the phosphorylated product is actually lecithin and not other phospholipids. The fat content of the liver does not change much in ordinary normal metabolism. The fat which comes there is worked over promptly and moved out. The phospholipid content changes even less and it is only under exceptional circumstances that there is a change in its percentage if calculated on a fat-free dry-weight basis. Even when there is a large accumulation of fat the phospholipid content if calculated on this basis remains remarkably constant (MacLachlan and Hodge 1939).

Large and "chronic" accumulations of fat are found in the liver under circumstances of which the following are the more important: 1, depancreatized dogs on certain mixed diets fed insulin (Fisher, 1924; Allan, Bowie, Macleod and Robinson, 1924; Best and Ridout, 1939). After a varying period of time there is an accumulation of fat and sometimes of cholesterol esters; 2, in high-fat, low-protein diets (Channon, Loach, Loizides, Manifold and Soliman, 1938); 3, after cystin feeding, but not after methionine (Tucker and Eckstein, 1938); 4, after cholesterol feeding (Okey and Yokela, 1936; Loizides, 1938); 5, fasting, if there is available fat in the depots; 6, anterior pituitary extract (Best and Campbell, 1938); and 7, in the hen at the onset of sexual maturity

(Lorenz, Chaikoff and Entenman, 1938). The substance which works best in removing or preventing the accumulation of fat in the liver is choline. Various other substances have been tried, e.g., derivatives or chemical relatives of choline such as betaine, creatin, choline methyl ester, tetra (β-hydroxy ethyl) ammonium chloride (Platt, 1939; Best and Ridout, 1939) and an alcoholic extract of pancreas (Dragstedt, Van Prohaska and Harms, 1936) known as lipocaic, about which findings differ as to details. Best and Ridout (1938) and MacKay and Barnes (1938a) reported that there was no active material in the lipocaic except choline and factors associated with protein, while Channon, Loach and Tristram (1938), after extensive experiments on rats, came to the conclusion that there was present a lipotropic factor in addition to choline and protein. Ralli, Rubin and Present (1938) found that if the pancreatic ducts were tied off but the pancreas left in place there was the same accumulation of fat in the liver as in depancreatized dogs. Montgomery, Entenman and Chaikoff (1939) found also an accumulation of fat in the livers of dogs after tying off the pancreatic duct if time enough was allowed (12–24 weeks). The formation of fatty livers was independent of weight loss and could be prevented by feeding raw pancreas. With increase of fat in the liver there was increase of cholesterol esters, but phospholipid content was not affected. Apparently something which prevents fatty livers is supplied by the pancreas, either as internal or external secretion or more likely produced by the action of the pancreatic juice on the intestine or on the food.

Anterior pituitary extract caused an increase of fat in the liver,—greater in guinea pigs and mice than in rats (Best and Campbell, 1938) which was not affected by choline or lipocaic (MacKay and Barnes, 1938b). Choline had no effect on the fatty infiltration following partial hepatectomy, an infiltration which was prevented by adrenalectomy.

The fact that choline is the most useful substance in the prevention and cure of fatty livers and the fact that colamine (aminoethyl alcohol) is not effective points to lecithin rather than cephalin as the important metabolic intermediate in the metabolism of fat in the liver and therefore probably in the whole organism. This idea is well supported by the evidence regarding phosphorylation of fat and by the lecithin-cephalin composition of the blood and tissues. Thus blood plasma is found to contain most of its phospholipid as lecithin (Williams, Erickson, Avrin, Bernstein and Macy, 1938; unpublished work by MacLachlan from this laboratory) while the corpuscles which take no important part in fat transport contain about 50 per cent cephalin and the rest lecithin

and sphingomyelin. In the liver the ratio is 60 lecithin to 40 other phospholipids; in muscle it is 55 lecithin to 45 cephalin; in brain 40 lecithin to 60 cephalin. Chargaff (1939) found that lecithin is synthesized in much larger amounts by the liver and intestine than cephalin. Sinclair's (1935b) hypothesis of a metabolic and a structural or functional type of phospholipid is plausible and it appears at present as though lecithin was the metabolic phospholipid with cephalin and possibly sphingomyelin as the structural or functional form. That choline actually stimulates the phospholipid turnover in the liver was shown by Perlman and Chaikoff (1939a) using the radioactive isotope of phosphorus. After choline feeding there was an increased formation as well as a rapid removal of radioactive phospholipid in the liver of rats. By the study of the effect of a single dose of choline, it was found that the increased phospholipid metabolism began about 1 hour after feeding and was over about 10 to 12 hours after. The increase in phospholipid metabolism was proportional to the amount of choline (up to 30 mgm. per rat). Cholesterol when fed is a factor in the production of fatty livers. Perlman and Chaikoff (1939b) have shown that it does so by depressing phospholipid turnover. Ingested cholesterol depressed the new formation of phospholipid (labelled). Choline fed along with cholesterol, however, gave their highest values for phospholipid turnover. These writers emphasize the fact noted for other tissues, that the phospholipid content of the liver is constant while the phospholipid turnover is not constant. Laszt and Verzár (1936a) found that agents which prevent phosphorylation such as iodoacetate and adrenalectomy prevented fatty livers after phosphorus poisoning or fasting and also prevented emptying of the fatty liver, from which they concluded that the mobilization of fat in both places was accompanied by a phosphorylation.

Blixenkrone-Møller (1938) has brought evidence to indicate that the ketone body formation by the liver, which has been known for a long time, may assume proportions which entitle it to consideration among the important mechanisms of transport of fatty acid fragments on the way to combustion. Evidence is brought to show that the liver can form these substances in large amounts but does not burn them while the muscles can burn but not create them. He has calculated that the perfused livers of depancreatized cats form up to 30 times as much ketone bodies as are excreted and that these fragments of which the ordinary fat fatty acids can yield 4 or more are consumed by the muscles. Similar conclusions were reached by Denal and associates (1936) in feed-

ing experiments with fatty acids to rats. They found that as many 4-carbon fragments, transformable into ketone body acids were supplied as the length of the fatty acid chain allowed.

PHOSPHORYLATION AS A MECHANISM OF FATTY ACID TRANSPORT. The increase of phospholipid in the blood during fat absorption has been reported many times (Sinclair, 1937) so that its occurrence as part of the mechanism of fat transport is generally accepted. Regarding the place and time as well as the manner of its formation, considerable effective work has been done although some essential details are still missing. The suggestion of Bloor (1915) indicating its formation in the red blood corpuscles has in general not been supported and the observation of the increase of fat and to a less extent of phospholipid of the corpuscles during fat absorption is regarded as an adsorption phenomenon. Sinclair (1929) made the observation that during fat absorption the phospholipids of the intestinal mucosa changed their fatty acids to correspond with those being absorbed but did not change in amount. Verzár and Laszt (1934a, b) found that when oleic acid and bile salts were introduced into the intestine of the rat they were absorbed at a fairly constant rate which was not affected by glycerol or phosphate alone but was accelerated by glycerol and phosphate together and still more by glycerol phosphate (increase of 150 per cent). The absorption was inhibited by monoiodoacetate and phlorizin, substances known to inhibit the phosphorylation of hexoses. It was believed, therefore, that a phosphorylation of the fatty acids to phospholipid took place during fat absorption. The absorption of fat from the intestine was inhibited by the same agents and by removal of the adrenals, agents which are known to inhibit phosphorylation of the sugars. These results definitely implicate phospholipid formation in the processes of fat absorption (Verzár and Laszt, 1935). Administration of cortical extract restored normal fat absorption in adrenalectomized animals. Flavin phosphate (B_2) restored normal fat absorption in animals poisoned with iodoacetate (Laszt and Verzár, 1936b). Mobilization of fat and production of fatty livers in rats was inhibited by adrenalectomy and restored to normal by cortical extracts and by flavin phosphate and yeast. Althausen, Anderson and Stockholm (1939) threw an interesting light on the function of the adrenals in phosphorylation. Adrenalectomy, as always, markedly impaired the intestinal absorption of dextrose but the rate was restored to normal by salt (NaCl). Similar results were obtained in the absorption of fat by Barnes, Wick, Miller and MacKay (1939) who found fat absorption normal in adrenalectomized,

salt-fed rats. Apparently it was not the lack of cortical hormone which inhibited phosphorylation but the resulting loss of salt. Süllmann and Wilbrandt (1934) found phospholipid increased greatly in the intestinal lymph of rabbits during fat absorption so that the fatty acids in phospholipid combination constituted about $\frac{1}{5}$ of the total fatty acids. Most of the absorbed fatty acids were, however, in the form of fat so that they must have been changed to neutral fat at some stage in their absorption. The high value for the phospholipid in the intestinal lymph found by Süllmann and Wilbrandt is apparently not found in thoracic duct lymph, the phospholipid values being ordinarily very much lower. Thus, during fat absorption, the phospholipid content of the thoracic duct lymph was increased (30 per cent) although relatively little in comparison with the neutral fat increase (400 per cent) (Brockett, Spiers and Himwich, 1934). The proportionate increase of fatty acids in phospholipid to fatty acids in neutral fat was about 1 to 22 so that only about 5 per cent of the extra fatty acid was carried as phospholipid.

Using an easily identifiable fatty acid, elaidic acid, Sinclair (1935) in addition to and in support of what he had shown previously was able to show that there are in the liver probably two types of phospholipid: the structural or functional and the metabolic. The structural phospholipids contain characteristically the more highly unsaturated acids, linoleic, arachidonic, and clupanodonic and are used up only by the processes of wear and tear, while the metabolic phospholipids contain the ordinary food fatty acids and serve in the transport and combustion of the fatty acids of the food fat. This second class exhibits a rapid turnover and when the intake of fat is great may show increases in amount (Artom, 1933b; Sinclair, 1935b). It is mainly into this part of the liver phospholipids that elaidic acid and presumably other food fat enters. This part of the phospholipid is being constantly given off to the blood and carried to the rest of the body where it is presumably burned. That this part of the phospholipid is metabolic rather than structural in most tissues (e.g., muscle and brain) is shown by the slow rate at which food fatty acid enters and leaves these phospholipids as compared with the rate at which it enters and leaves the phospholipids of the liver, the intestinal mucosa and to a less extent the kidney.

These results were confirmed and extended by a series of experiments by different investigators using radioactive phosphorus, P^{32}, which entering the phospholipid during its synthesis served as a tracer in following its progress. Artom, Sarzana and Segré (1938) fed radioactive phosphorus to rats on carbohydrate and fat diets. In both cases

the amount of active phosphorus in the tissues varied with the tissue, and fat in the diet accentuated the difference. Liver and intestine had the highest values; then kidney, lungs and spleen; and finally the heart, testicles and muscles with nervous tissue lowest. In long-continued experiments, the difference faded out, indicating a general distribution with time. Ingested fat increased the synthesis of phospholipid in the liver and intestine while in other tissues the difference though present was slight, indicating that it was probably secondary, the result of absorption from the blood which transports the newly synthesized phospholipid. In the cells of the intestinal mucosa most of the newly formed phospholipid is transformed into fat again, but some of it may pass into the blood. Synthesis of radioactive phospholipid from inorganic phosphorus has been accomplished in the surviving perfused liver by Hahn and Hevesy (1938) and in intestine, liver and kidney slices by Robinson, Perlman, Ruben and Chaikoff (1938). Chaikoff and associates (Fries, Ruben, Perlman and Chaikoff, 1938) by the use of radioactive phosphorus have shown that tissues vary greatly in their phospholipid turnover. Three tissues, the intestinal tract, the liver and the kidney, were particularly active showing a maximum content of the radioactive phospholipid in from 6 to 12 hours after ingestion of the labelled phosphate. Other tissues showed a slower rise which continued for many hours, e.g., in the brain for at least 200 hours, followed by a slow decrease. At 800 hours the brain was still 70 per cent of what it was at 200 hours (Changus, Chaikoff and Ruben, 1938). This picture of phospholipid activity is very similar to that obtained by Sinclair using elaidic acid and indicates that these three tissues regularly form phospholipid from ingested fat and that this phospholipid is later carried to the other tissues, constituting an important part of the mechanism of fatty acid transport. The formation of phospholipid by liver and intestine takes place even in fasting, indicating that the stored fat when mobilized undergoes the same processes as the fat absorbed from the intestine. Judged by the evidence from fatty livers (see p. 9) an adequate exogenous supply of choline seems to be essential for continued phospholipid formation and hence for the disposal of fat and cholesterol esters.

The participation of the kidney in the rapid changes in the phospholipid during fat absorption or mobilization as shown by Sinclair and by Chaikoff and associates and confirmed in unpublished work by Haven from this laboratory is an unexpected development which lacks any adequate explanation. It is known that the kidney excretes phosphate

as part of the mechanism for the removal of excess acid and as part of its base-sparing activity, and it is possible that the phospholipid takes part in that process. Support for this conception is furnished by unpublished work of Weissberger from this laboratory. Using radioactive phosphorus along with ammonium chloride, she has been able to show an increased turnover by the kidney of phospholipid as well as inorganic phosphate. The fact noted by Griffith and Wade (1939) that choline deficiency results in hemorrhagic degeneration of the kidneys adds weight to this idea and offers an indication that phospholipids take part in all processes involving phosphoric acid.

The major part of the phospholipid turnover by the gastrointestinal tract is performed by the small intestine, the stomach and large intestine being relatively unimportant. The removal of the gastrointestinal tract and kidneys does not markedly influence the phospholipid.

TRANSPORT ACROSS THE PLACENTA AND EGG MEMBRANES. *Placenta.* Sinclair (1933) showed that the placenta of the rat is permeable to the highly unsaturated acids of cod liver oil as it is also to elaidic acid (McConnell and Sinclair, 1937); when trielaidin was fed to the mothers, the new-born young contained 16 per cent of their liver phospholipid fatty acids and 11 per cent of their whole body fatty acids as elaidic acid. After 10 days' suckling, the body fatty acids had risen to 61 per cent elaidic acid and the liver phospholipid fatty acids to 27 per cent, showing a ready passage of this fat into the milk. Bickenbach and Rupp (1931) found that the placenta of the rabbit was permeable to the unsaturated acids of linseed oil. Sinclair is careful to note that the placentas of the rat and rabbit are of the simplest type, only the endothelium of the fetal blood vessels separating the fetal from the maternal blood, a fact which made it unwise to conclude that the more complicated human placenta is permeable to lipids. However, Boyd and Wilson (1935) were able to supply some of the necessary proof. These workers found that the human placenta at full term adds considerable lipid to the umbilical blood from which it is absorbed by the fetus. The average large, well-nourished infant was found to absorb about 50 grams of lipid per day from umbilical blood, 40 grams being phospholipid and the remainder equally divided between free cholesterol and cholesterol esters. Two possible explanations were offered regarding the manner in which this transfer took place. First, the lipids may be simply passed across the placental membrane to the umbilical blood, and second, the placenta may act as a secreting gland taking up lipids from the maternal blood stream and passing them on, with or without modifi-

cation, to the fetal circulation. Later work by Boyd (1935) with rabbits supported the second explanation, i.e., that the tissue substance of the placenta was actively engaged in the transfer of at least a part of the lipid.

The hen's egg. The entire food of the growing chick embryo is contained in the surrounding egg and largely in the immediately adjacent yolk. The yolk consists mainly of lipids made up of about $\frac{2}{3}$ fat and cholesterol and about $\frac{1}{3}$ phospholipid. Much of this material is used up during the growth of the chick. Are these materials absorbed by simple diffusion through the fetal membranes or is there a process of digestion and absorption or secretion involving making over and modification of the yolk lipids in the transfer from food depot to living tissue? The phospholipid which appears in the embryo is apparently new phospholipid and not the phospholipid of the egg yolk. Hevesy, Levi and Rebbe (1938) introduced radioactive phosphorus as phosphate into the egg before incubation and then examined its distribution after 6, 11, 16 and 18 days. They found the phospholipid in the embryo highly active while that of the yolk was inactive, indicating that the embryo synthesized its phospholipid anew. Kugler (1936) found that lecithin and cephalin values paralleled each other both in yolk (decrease) and in embryo (increase) preserving the initial ratio of 3 lecithin to 1 cephalin, which means according to the results of Hevesy and associates that these compounds were resynthesized in the embryo in the same proportions as in the yolk. They need not be the same phospholipids, since Eaves (1910) showed that the embryo after about the tenth day can desaturate fatty acids. Hanes (1912) found that cholesterol esters began to accumulate in the liver of the developing chick about the fourteenth day of incubation, at which time a large movement of fatty material from the yolk to the embryo took place. Since there was only free cholesterol in the yolk, this must have been transported into the embryo and combined with the fatty acids at about the time that there was a large breakdown of phospholipid. Hanes' conception of the chain of events as regards the fatty acids was as follows: 2 phospholipid + cholesterol = fat + cholesterol ester.

CHOLESTEROL ESTER AS A FATTY ACID CARRIER. Two-thirds or more of the cholesterol of the plasma is combined with fatty acids as esters which means that, in terms of average values, about 100 mgm. per cent or $\frac{1}{4}$ of the total fatty acids or about $\frac{3}{4}$ of the value for the neutral fat of the plasma is carried as cholesterol esters. Boyd (1937) in presenting a study of a wide range of plasma lipid values showed that neutral fat,

cholesterol esters and phospholipid followed an almost parallel course from the lowest values to about the upper limit of the normal range of total lipid. Beyond that point neutral fat increased faster while phospholipid continued to increase linearly with total lipid. Cholesterol ester after a temporary falling off resumed its increase parallel with the phospholipid. These results indicate that cholesterol ester is nearly as important as phospholipid in fatty acid transport. Of the three main fatty acid compounds of blood plasma,—neutral fat, phospholipid and cholesterol esters, the degree of unsaturation was found to be greatest for the cholesterol ester fatty acid, next for the phospholipid and lowest for the neutral fat (Bloor, Blake and Bullen, 1938), indicating a special function of the cholesterol ester combination with respect to the unsaturated fatty acids of the blood plasma. MacLachlan (1936) found that in ungerminated seeds (soy beans) 85 to 90 per cent of the sterol was in the free form, while in the cotyledons of the germinating seed (i.e., the place where food material for the young plant is being prepared) about 60 per cent of the cholesterol is esterified with fatty acids.

Very little cholesterol ester is found elsewhere in the body than in the blood. Most of the cholesterol of the normal tissues is in the free form, the percentage of cholesterol as ester never being more than 10 per cent and generally below 2 per cent (Thaysen, 1914). Exceptions to this rule are the adrenals in which there is often much cholesterol ester. Under certain circumstances fatty livers may also contain considerable cholesterol ester. The fact that cholesterol in normal tissues is almost entirely in the free form leads to the inference that the cholesterol esters of the blood when they pass into the tissues are deprived of their fatty acids which are then used for the purposes of the cells. The avidity with which the cellular phospholipids take up the more highly unsaturated acids suggests the possibility that they get their fatty acids from the cholesterol esters. Cholesterol is not burned to any appreciable extent in the tissues but with the phospholipids constitutes a balanced unit which is part of the structure of the cell.

Deposition of cholesterol esters in the liver takes place under certain conditions, e.g., 1, feeding cholesterol together with fat (Beeston, Channon and Wilkinson, 1935; Okey and Yokela, 1936). Increasing the protein in the diet increases the deposition (Beeston, Channon and Wilkinson, 1935). 2. In depancreatized dogs fed insulin, there is storage of cholesterol esters along with fat in the liver, accompanied by a disappearance of cholesterol esters from the blood (Chaikoff and Kaplan, 1935). Feeding of choline in these cases causes the removal of the

fat and, with more difficulty, the cholesterol esters, but does not bring back the cholesterol esters to the blood. Feeding raw pancreas, however, does bring the blood cholesterol ester back to its normal level.

Cholesterol is not well absorbed except in the presence of fat. During the absorption of fat, the cholesterol ester content of the blood increases (Knudson, 1917). The continuance of cholesterol esters in the blood appears to depend on the normal condition of the liver (Epstein, 1931) and on the presence of the pancreas or its secretions (Chaikoff and Kaplan, 1935). The balance between free and esterified cholesterol in the blood appears to be maintained by the enzyme cholesterolesterase which has been studied by Sperry and Stoyanoff (1937). They found that there was a marked esterification of free cholesterol on incubation of untreated serum, which did not take place if the serum had previously been heated to 55–60°C. for 1 hour. In human serum the esterification was inhibited by bile salts so that there was no significant change in the proportions of free and bound cholesterol during incubation. In dog serum, on the other hand, the larger amounts of sodium glycocholate brought about complete hydrolysis of the cholesterol esters; with smaller amounts the hydrolysis decreased until a level was reached at which the reaction was reversed and some esterification occurred as in human serum. Klein (1938) reported a marked hydrolysis of cholesterol esters of serum at pH 5.3 by saline extracts of tissues. Riegel, Ravdin and Rose (1937) found that incubation of plasma with normal dog or human hepatic bile caused hydrolysis instead of esterification as occurs when plasma is incubated alone. Eckstein (1938a, b) found that rats synthesized more cholesterol on a high-fat diet than on a low-fat diet which might be interpreted as the result of a greater need for cholesterol in handling the extra fat. It was also shown that the increase of cholesterol was greater with an unsaturated fat (iodine no. 118) than with a saturated fat (iodine no. 8), emphasizing the relation of cholesterol to unsaturated acids mentioned above. Cook (1938) from a study of the lipid balance sheet of fecal excretion concluded that the metabolism of cholesterol was related to the increase of fatty acids in the feces, since the gain in fatty acids was approximately the same as the loss of cholesterol, as if the missing cholesterol (20–30 per cent of the amount fed) had been transformed into fatty acids. He found that cholesterol was not absorbed on a fat-free diet. Brockett, Spiers and Himwich (1934) found a marked rise in cholesterol both free and combined in the thoracic duct lymph during fat absorption. The increase amounted to about 200 per cent of the value in

fasting lymph, which led them to the conclusion that cholesterol aids in the absorption of fatty acids. Esterification occurred in the intestinal mucous membrane.

TRANSPORT AS NEUTRAL FAT. The presence of fat in the blood in the form of very fine particles has been known for a long time. These particles are mostly from $\frac{1}{2}$ to 1 μ in diameter, are known to originate mainly from the intestine by way of the chyle, and are recognized as fat in process of transport. Most of the fat absorbed from the intestine reaches the tissues in this form and is removed and stored as fat by a process of which the details are unknown. When the removal process is interfered with, the fat particles accumulate in the blood producing the condition known as lipemia. The essential feature of this condition is the increase in the finely suspended fat which is however accompanied or followed by relatively small increases of the other blood lipids,—phospholipid, cholesterol and cholesterol esters. During fasting and especially with exercise (Gage and Fish, 1924) there is a mobilization of fat in this same finely divided form, and presumably whenever fat is mobilized from the stores it assumes this form. Barrett, Best and Ridout (1938) using heavy hydrogen, D_2, as a labelling agent were able to show that the neutral fat which accumulates in the liver originates in the fat depots: 1, in fasting; 2, after anterior pituitary extracts, and 3, after carbon tetrachloride poisoning in mice, while after a high-carbohydrate, low-protein diet or after a high-protein diet the liver fat is not the fat of the depots but is apparently that formed from carbohydrate or the protein. While the mobilized fat is known to proceed to the liver, the recent investigations of Ivy and associates (Rony, Mortimer and Ivy, 1933) indicate that it may also go to the intestine since the thoracic duct lymph in dogs fasted 2 to 14 days was found to contain 0.25 to 1.3 per cent of total fatty acids while the average content 24 hours after a fat meal was 0.2 per cent. Since there was no increase after removal of the intestine, this extra lipid must have been derived from the intestine and must have come to the intestine from the fat stores.

The purpose of mobilization of fat to the liver and to the intestine has until recently not been known, although various hypothetical explanations have been offered, such as the desaturation and phosphorylation hypothesis of Leathes. Work reviewed in this paper (see phosphorylation) shows that both liver and intestine do phosphorylate fat, i.e., change it to phospholipid, and it is therefore possible that the mobilization of neutral fat to the liver and intestine is mainly for this

purpose. During the process, there is probably a rearrangement of the fatty acids in the glyceride molecule to produce the standard tissue phospholipid pattern of about one saturated acid to three unsaturated acids per double molecule noted by Bloor (1928) and by Sinclair (1935a). Since the esterification of cholesterol with the fatty acids is known to occur during absorption from the intestine, another reason for the mobilization may be the esterification of cholesterol.

A study of the tiny fat particles (chylomicrons) in blood has been made by Ludlum, Taft and Nugent (1930) who think that the fat globules are stabilized by a protein film, since the first zone of aggregation of the particles occurs at a pH between 4.7 and 5.3, which are approximately the isoelectric points of albumin and globulin, respectively, and coalescence takes place when the acid is strong enough to precipitate the protein and destroy the film. Palmer and Wiese (1933) found that the membrane surrounding the globules of fat in milk was composed of a mixture of protein and phospholipid of which the protein was not the same as the other milk protein and the phospholipid consisted of both mono and diamino phospholipids. The relation between protein and phospholipid was not constant. The membrane was removed by churning.

Material for the formation of milk fat and probably also of egg fat is apparently transported as neutral fat, since other means of origin of the milk fat from lipids seem to be ruled out. The work of Gage and Fish (1924) with stained fat indicates that in carnivorous animals the transfer of fat from blood to milk is immediate and rapid while in the herbivorous animals the transfer is much slower. The interchange of neutral fat through the human placenta is noted by Boyd (see under placenta). The claim by Meigs and associates (1919) that blood phospholipid is a source of milk fat seems to be refuted by the work of all later investigators (Graham, Jones and Kay, 1936; McCay and Maynard, 1935). The idea that cholesterol esters of blood serve as a source of the unsaturated fatty acids of milk was advanced by Schaible (1932) who reported that the amount of unsaturated acids in cholesterol esters was greater than that in the phospholipid. The blood of lactating cows has a much higher lipid content than that of non-lactating cows or steers and the increased value is mainly in the neutral fat and phospholipid (Schaible, 1932). Maynard, McCay and Madsen (1936) found that the degree of unsaturation of the blood lipids and milk fat followed closely changes in the degree of unsaturation of the food fat. After the shift in dietary fat, there was a marked change in the iodine

number of the milk fat in half the cases in 18 hours and in all the cases within the next 24 hours.

The fat of egg yolk appears also to be food fat deposited after transport as fat. The evidence on this point is, like that for milk, largely indirect. Gage and Fish were able to trace stained food fat directly and promptly to the fat of the yolk of laying hens. Chaikoff and associates (Lorenz, Entenman and Chaikoff, 1938) showed that the lipids (all lipids but cholesterol esters) of the blood of laying hens were very much higher than those of non-laying or immature hens or of roosters. By far the greatest increase was in the neutral fat. Similar increases were found in the neutral fat of the liver (Lorenz, Chaikoff and Entenman, 1938) at sexual maturity.

The passage of lipids through capllaries has been considered by Süllmann and Verzár (1934). They found that membranes which would allow water blue and congo red to pass and which were similar in permeability to blood capillaries would permit the diffusion of 25 per cent of the lipids from lipemic blood. All fractions passed equally well. Marble, Field, Drinker and Smith (1934) found that injection of chyle or of fat emulsions into the blood stream of dogs resulted in a slight rise in the total lipid of lymph with a smaller rise for cholesterol. They believed that the permeability of the blood vessel wall for lipids was slight but definite. On the other hand, Man and Peters (1933) investigating the effect of standing still for ½ hour on the content of lipids in the blood serum, a procedure which is known to cause a decrease of blood volume and an increase of serum proteins of about 10 per cent, concluded that the capillary walls were ordinarily impermeable not only to proteins but also to cholesterol, phospholipids and compounds of saturated and slightly unsaturated fatty acids. At the same time they realized that the distribution of lipids to the tissues must involve their passage through the capillary walls in spite of this demonstrated impermeability. It should be noted that their experiments were done on individuals in the post-absorptive state in which probably most if not all the excess lipids had already passed out of the blood leaving only those which were necessary constituents of the serum and possibly combined in some way with the proteins. Changes in the permeability of the capillaries should also be taken into account.

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